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Cerium oxide nanoparticles influence the life cycle of spontaneous plant species

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CONTENTS

ABSTRACT

The tumultuous development of nanotechnology, a new emerging field of science, and the consequent increasing use of engineered nanomaterials (ENMs) in different products and applications is considered a potential threat not only for human health but also for the environment. Studies regarding the impact of such materials on living organisms and biota and their potential transfer through the food web are analyzed in very few studies and are still at an early stage. Released ENMs could accumulate into environmental compartments where they could establish complex interactions with different abiotic and biotic components, especially in terrestrial ecosystems. Different studies regarding ENMs, focus on crops, but the effects of these new materials on common and spontaneous species are quite completely unknown and poorly investigated. This aspect could be very important in view of a future possible Ecological Risk Assessment. Cerium oxide nanoparticles (*n*CeO_2) are one of the most widely utilized ENMs in Europe and have a great potential to accumulate and affect the environment because of their widespread applications in commercial products. The remarkable and rapid increase in the use of CeO_2 nanoparticles in many application areas caused the spread of these materials even in the different environmental matrices. For example, when *n*CeO_2 reaches the soil, it could be absorbed by vegetation, sinks deeper into the soil layers, contaminating aquatic groundwater, or it could also enter into the food chain. A germination experiment and a pot soil study were carried out in order to observe the response of three spontaneous plant species (*Holcus lanatus* L., *Diplotaxis tenuifolia* L. DC. and *S. flos cuculi* L.) to different concentrations of *n*CeO_2 having different dimensions (25 nm and 50 nm, respectively). Germination and root elongation, plant growth parameters and

nCeO_2 uptake and bioaccumulation were investigated. In the three species, treatments with *n*CeO_2 increase the percentage of germination and stimulate root elongation in the first stages of plant growth. Moreover, treatments increase the development of roots if it is compared with control plants and the same comparison could be done for leaf area, with higher values in treated plants than in control ones. ICP – MS analysis highlights that, at the same high concentration of *n*CeO_2, plants absorb a greater quantity of 25 nm particles than 50 nm, because *n*CeO_2 50 nm tend to agglomerate and their uptake becomes more difficult.

Key words: cerium oxide nanoparticles, environmental impact, spontaneous species, germination, biometric variables, cerium uptake.

1. INTRODUCTION

1.1 Nanoparticles and nanotechnology

Nanotechnology could be considered a relatively new process of control and manipulation of structures at the nanoscale, but nanomaterials (NMs), though unknown, were also present in ancient times: for example, metallic nanoparticles were in ceramics (Caiger – Smith, 1991) and copper nanoparticles in the red glass of Roman mosaics and in Celtic red enamels (Brun et al., 1991; Colomban et al., 2003; Ricciardi et al., 2009). At that time, ancient civilizations did not understand the particular properties and the real potential of nanoparticles (NPs). The first time that the concept of nanotechnology was really described was in 1959, when the Nobel Prize winner in physics, Richard Feynman, tried to face the problem of manipulating matter on an extremely small scale. “We are working with different laws and we expect different things. At the atomic level, we have new kinds of forces and new possibilities, new kind of effects” (Feynman, 1959). With this phrase, he suggested the possibility of manufacturing objects and highlighted possible important technological improvements, connected to the rearrangement of atoms. But he also understood the difficulties of manipulating these small materials with their particular behavior and properties. Nanotechnology could be defined as the specific research that has NMs as reference and the take up of atoms, molecules and macromolecules (figure 1). In the last few years, nanoscience and nanotechnology have been rapidly expanding and are recently emerging as one of the most important and developed scientific and technological sectors (Maynard et al., 2006).

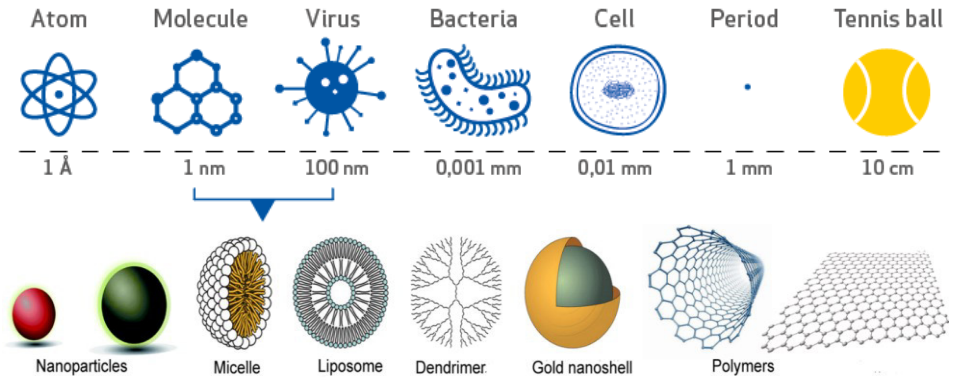


Figure 1: Nanomaterials (NMs) and "where" they could be positioned in the "biological world".

NPs are becoming an important part of society. Moreover, there has been an increase in investment in nanotechnology research, not only in the United States, but also in Western Europe and in Japan (Nowack and Bucheli, 2007; Keller and Lazareva, 2014). Nowadays in the European market there is already a large number of products containing NMs, such as batteries, antibacterials, clothing, cosmetics and food products. According to several experts, nanoscience and nanotechnology are rapidly evolving in different applications, with the potential to revolutionize modern life; for this reason, nanotechnology is considered to be the basis of the next technological revolution: the so called Nano Revolution (Ramsden, 2005). Nanotechnology is also one of the EU Key-Enabling Technologies (KETs) (Meesters et al., 2016) that contribute to the development of new machines and new products and the way by which materials can be developed to solve many problems in medicine, engineering, agriculture, biology, chemistry, surface science, space exploration, ocean and marine science, geography and geology (Meesters et al., 2016).

1.2 Nanoparticles characteristics

The small scale of nanoparticles (NPs) gives them a specific, unique and beneficial characteristics such as physical, chemical, electronic, electrical, mechanical, magnetic, thermal, dielectric, optical and biological properties, which sometimes are opposed to the relative bulk materials (Schmid, 1992; Daniel and Astruc, 2004). Fundamental aspects that characterize NPs are: size and surface area, composition, shape and concentration. Particle size and surface area play a major role in interaction of materials with biological and environmental systems. A decrease in size of the materials means an exponential increase in surface area relative to volume, creating a more reactive surface of nanomaterials (NMs). It has been demonstrated that different biological and chemical mechanisms are dependent on the size of material (Nel et al., 2006; Aillon et al., 2009). In general, the particle size defines the toxicity of NPs, which can be attributed to its ability to enter into biological and environmental systems (Lovric et al., 2005), interfering for example, with plant biological functions. Furthermore, several studies analysing the different classes of NPs showed that surface area is also another important factor (Holgate, 2010). The small size of NPs corresponds to high surface area (Risom et al., 2005; Donaldson and Stone, 2003). Several studies demonstrate that smaller NPs of dimensions < 100 nm cause adverse effects not only in human beings but also in plants, if compared to larger particles of the same material (Donaldson et al., 2003; Gurr et al., 2005). As a result, the translocation or distribution of NPs has been found to be size dependent. Although size and surface area are important factors that characterised NPs, there are other features, such as chemical nature and shape, that give unique properties to these materials. Shape is another characteristic that influences

biological and chemical processes (Verma and Stellacci, 2010; Chen et al., 2009) and different studies demonstrate that if carbon nanotubes, silica, nickel, gold, and titanium NMs have a spherical shape, they are more easily absorbed by cells than NPs that have irregular shapes (Petersen and Nelson, 2010; Ispaset al., 2009; Chitihrani et al., 2006; Hamilton et al., 2009; Champion and Mitragotri, 2006). Although it has been emphasized that particle size and shape play a significant role, for example, in deciding the toxicity of NPs, it is important to highlight that NPs, having the same dimensions, could have different effects, due to chemical properties and composition. For example, a study conducted by Griffitt et al., (2008) has demonstrated that silver and copper NPs with the same dimensions have different effects and different toxicity levels. The aggregation state influences NPs properties but it is due to a mix of other characteristics cited above. Indeed, the aggregation state depends on size, surface charge and composition. Some NPs could have more adverse effects if they are agglomerated (Wick et al., 2007) and generally, it has been observed that with an increase in concentration, the toxicity of NPs decreases.

1.3 Engineered nanoparticles and their applications

Nanoparticles (NPs) can be divided into natural and anthropogenic particles. The existence of natural NPs in water, air and soil is well known from the beginning of Earth's history and have been recorded in 10,000 – year - old glacial ice cores (Murr et al., 2004); there is also evidence of natural NP formation in sediments of the Cretaceous (Elsila et al., 2005). However, NPs have been created by humans for millennia, deriving mainly from combustion (Buzea et al., 2007). Anthropogenic NPs could be either inadvertently formed

as a by - product, mostly during combustion, or produced intentionally due to their particular characteristics. More recently, the principal sources of anthropic NPs are represented by chemical manufacturing, welding, ore refining and smelting, combustion in vehicles and airplane engines (Rogers et al., 2005). It is important to underline that current research focuses attention on engineered nanoparticles (ENPs) and in particular on metal oxide NPs, because in recent times they are widespread in so many productive sectors and consequently could become a potential threat not only for human health but also for the environment. ENPs are commonly used in cosmetics, sporting goods, tires, stain-resistant clothing, sunscreens, toothpaste, food additives, etc. These nanomaterials (NMs) constitute a small minority of environmental NMs (Buzea et al., 2007). Nowadays ENPs can be divided into several classes, such as carbonaceous NMs, metal oxides, semi-conductor materials, zero-valent metals, quantum dots and nanopolymers (Bhatt and Tripathi, 2011). Metal oxide NPs include both individual ones (such as titanium dioxide (TiO_2), cerium dioxide (CeO_2), chromium dioxide (CrO_2), molybdenum trioxide (MoO_3), bismuth trioxide (Bi_2O_3) and binary oxides (such as, lithium cobalt dioxide ($LiCoO_2$)). Metal oxides have specific catalytic, optical and physical properties. ZnO NPs have emerged as one of the most promising oxide materials because of its numerous industrial applications in the fields of medicine, pigments, catalysts, ceramics, and rubber additives (Wang et al., 2010; Kumar and Khare, 2008). ZnO nanostructures also have potential applications in solar cells, electrodes, sensors, transparent UV protection films, UV light emission, surface acoustic waves, and magneto-optical devices (Wang et al., 2010; Kumar and Khare, 2008; Kumar et al., 2009; Zhuge et al., 2010). This wide range of applications are due to their electrical, optical, and magnetic properties (Al-Salman and Abdullah, 2013). TiO_2 NPs have been applied in photocatalytic

water splitting (Fujishima and Honda, 1972; Ni et al., 2011), purification of pollutants (Hashimoto et al., 2005; Pozzo et al., 1997; Carp et al., 2004; Fujishima et al., 2008), photocatalytic self-cleaning, photocatalytic antibacterial (Hashimoto et al., 2005; Fujishima et al., 2008; Blake et al., 1999; Fujishima et al., 2000) and photo-induced super hydrophilicity material (Hashimoto et al., 2005; Carp et al., 2004; Fujishima et al., 2000). TiO_2 NPs are also used in photovoltaic (Carp et al., 2004; Grätzel, 1999; Grätzel, 2001; Grätzel, 2005) and photosynthesis applications (Carp et al., 2004). TiO_2 NPs have been used in many applications because they provide increased surface area where photo-induced reactions may occur, enhancing the light absorption and photo - reduction rates and resulting in higher surface photoactivity. At the same time, the high surface-volume ratio of NPs increase the photocatalytic reaction rate (Lan et al., 2013). CeO_2 has long been employed as a bound catalyst in catalytic converters for diesel engines and it is now finding application as a NP additive to diesel fuels. Its use as a fuel additive has been associated with reduced fuel consumption and reduced emissions of combustion derived NPs and unburned hydrocarbons (O'Brien and Cummins, 2011). CeO_2 NPs have also recently gained a wide range of applications including coatings, electronics, biomedical and energy (Cassee et al., 2011): in particular, in glass polishing material (Bekyarova et al., 1998; Jiang et al., 1998); as a coating for corrosion protection for metals and alloys (Hamdy, 2006; Zhong et al., 2008); UV-blockers and filters (Tsunekawa et al., 2000; Yamashita et al., 2002); high temperature oxidation resistant coating (Patil et al., 2002); sunscreens (Masut et al., 2000); as an additive to glass to protect light-sensitive material (Lan et al., 2013) or in ceramics (Bhaduri et al., 1988; Messing et al., 1993); as an oxidation catalyst (Yakimova et al., 2009), in the energy industry as an oxygen ion conductor in solid oxide full cells (Yahiro et al., 1988), electrolyzers (Inaba and Tagawa,

1996), oxygen pumps, amperometric monitors (Hirano et al., 1996), solar cells (Corma et al., 2004), photocatalytic oxidation of water for the generation of hydrogen gas (Bamwenda and Arakawa, 2000; Chung and Park, 1996); and as an anode material for lithium ion battery systems (Zhou et al., 2007). CeO_2 NPs can be used in the biomedical field as protection of primary cells from the detrimental effects of radiation therapy (Tarnuzzer et al., 2005), neuroprotection to spinal cord neurons, prevention of retinal degeneration induced by intercellular peroxide (Das et al., 2007), potent antioxidant in cell culture models (Patil et al., 2002) thanks to free-radical scavengers (Babu et al., 2007). CeO_2 NPs can be used as slurry in semiconductor fabrication (Jiang et al., 1998), as buffer layers with silicon wafers (Tashiro et al., 2002) or gates for metal-oxide semiconductor devices (Galata et al., 2007). Other utilizations of CeO_2 NPs are: high temperature oxidation safe guards (Zhou et al., 2007) and gas sensors (Stefanik and Tuller, 2001), low-temperature water gas shift catalysts (Hilaire et al., 2001), removal of organics from wastewater (Matatov-Meytal and Sheintuch, 1998), photo degradation of toluene in the gas phase (Hernández-Alonso et al., 2004), photocatalytic behavior under sunlight irradiation to degrade dyes (Zhai et al., 2007; Borker and Salker, 2007). CeO_2 NPs have been used in this wide range of applications because they show absorption properties in the UV, low photocatalytic antioxidants properties, high thermal stability (Trovarelli et al., 1999), facile electrical conductivity and diffusivity (Zhou et al., 2007), high degree of hardness, specific chemical reactivity (Chen and Chang, 2005), ability to store and transport oxygen as large oxygen storage capacity (Shahin et al., 2005) and high refractive index (Goharshadi et al., 2011).

1.4 Nanoparticle analysis and characterization

In the last few years, nanoscience and nanotechnology have been swiftly evolving in different applications, becoming an area of rapid development with the potential to revolutionize modern life and to provide a route to environmental contamination (Aitken et al., 2006; Roco, 2005). Nanoparticles (NPs) and nanomaterials (NMs), as mentioned before, could enter in contact with people and interact with ecosystems, so it is essential to characterize these new materials, following correct protocols and measurement approach and with precise instrumentation and analytical methods (Holt et al., 2000). The correct NP analysis and the methodology of characterization is an important step to arrive at a possible future environmental risk assessment of engineered nanoparticles (ENPs). In table 1, there are reported the NPs characteristics with the applicable analytical technologies, but it is important to underline that in the present dissertation, it will be briefly described the main techniques that are used for NPs characterization. As regards the analytical methodologies used in the context of chemical qualitative and quantitative characterization of NPs, particular attention is referred to the application of Inductively Coupled Plasma - Mass Spectrometry techniques (ICP-MS) (Montaser, 1998), that is one of the leading analytical techniques capable of measuring and assessing many of these key characteristics of metal - containing particles. It is characterized by the combination of an instrument for plasma emission spectroscopy with a detection system for mass spectrometry. It allows qualitative and quantitative determinations of the components of metal based NMs, metal oxides NPs and ENPs. Moreover, through the coupled techniques of liquid chromatography (High Performance Liquid Chromatography, HPLC) with systems of mass spectrometry detection (HPLC-

MS), it is possible to carry out a characterization of the impurities due to organic compounds with different degrees of volatility, if they are present in the engineered nanomaterials (ENMs).

Table 1: Nanomaterial characteristics and applicable analytical technologies (Salamon, 2010)

Analytical Technique	Nanomaterial Characteristics							
	Concentration	Particle Size	Surface Charge	Surface Area	Shape	Agglomeration	Structure	Composition
Inductively Coupled Plasma - Mass Spectrometry	X							X
Single Particle ICP - MS	X	X				X		X
Liquid Chromatography / Mass Spectrometry	X							X
Optical Spectroscopy - UV / Vis	X							X
Fluorescence Spectroscopy	X	X				X		X
Scanning Electron Microscopy		X			X	X	X	
Transmission Elecron Microscopy		X		X	X	X	X	X
Confocal Microscopy		X			X	X	X	
Dynamic Light Scattering		X			X	X		
Static Light Scattering		X			X	X		
Laser - Induced Plasma Spectroscopy		X						
Ultrafiltration		X						
Centrifugation		X				X		
Filtration		X						
Hydrodynamic Chromatography		X						
Selected Area Electron Diffraction		X					X	
Zeta Potential by DLS			X					
Molecular Gas Absorption (BET)			X	X				
X - ray Diffraction							X	

The morphological analysis of NMs can be performed using electron microscopy techniques scanning (SEM) and scanning of probe. Scanning electron microscopy (SEM, Goldstein, 1981) allows to obtain information on the morphology of the NMs (shapes and sizes) with resolution lower than 50 nm. Another important technique is the transmission electron microscopy (TEM, Williams, 1996), that allows to obtain structural information with lower resolutions at the nanometer. Furthermore, TEM through the diffraction of electrons, in its own different modes (Selected Area Electron Diffraction (SAED) and nanodiffraction), and the high resolution (High Resolution TEM (HRTEM)) allows to study the crystal structure of NMs, also highlighting the presence of amorphous material. Nevertheless, the best technique for studying the crystal structure is the X – ray Diffraction (XRD). In addition, information on the composition of NM elements could be obtained through the Energy Dispersive X - ray Spectroscopy (EDXS).

1.5 Nanoparticles in the environment

In the last few years, nanoscience and nanotechnology are rapidly evolving in different applications, becoming an area of rapid development with the potential to revolutionize modern life and to provide a route to environmental contamination. Nanoparticles (NPs) and nanomaterials (NMs) could enter in contact with people and interact with ecosystems. These new materials derive from both natural sources, such as volcanism, meteoric dust and organic matter in soil and water (Pan and Xing, 2012; Rietmeijer and Mackinnon, 1997; Akaighe et al., 2011) and anthropogenic activities, as manufacturing processes of different industries. In particular, large quantities of synthetic

nanotechnology materials are produced: it is estimated that tons of TiO_2, CeO_2, FeOx, AlOx and ZnO are causing a big discharge of NPs and NMs into natural ecosystems (Piccinno et al., 2012; Gottschalk and Nowack, 2011) from point and diffuse sources that contaminate atmosphere, soil and water (Cassee et al., 2012). A scheme of the possible interaction between engineered nanomaterials (ENMs) and the environmental matrices is here represented (figure 2).

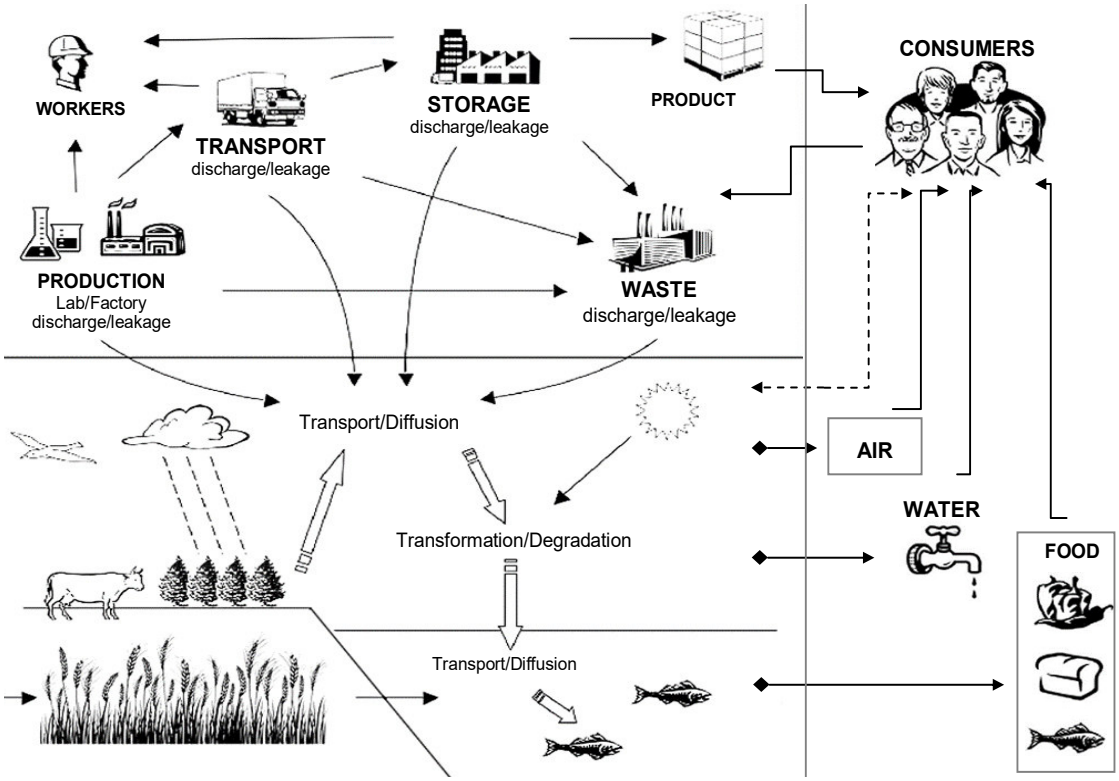


Figure 2: Envronmental fate of engineered nanomaterials (ENMs) (Meesters et al., 2016).

The progressive development in the knowledge of NMs regarding their advantages and potential uses does not correspond to the advance of analysis

about their possible environmental destinations and effects on ecosystems and consumers, in particular because of the complex interactions between NMs with abiotic and biotic components (Lawrence et al., 2016; Tourinho et al., 2012; Mueller et al., 2009). The global production of NMs inevitably causes the accumulation of these materials in soil and water systems and the interaction with biota. Indeed different studies highlight the presence of NMs in sediments and soil but also in plant roots. Indeed, NPs could accumulate in roots (Gardea - Torresdey et al., 2014) and could be transferred along trophic levels if they are consumed by soil organisms, such as arthropods, annelids, insects and other species (Kim et al., 2016; De la Torre Roche et al., 2015). Regarding the relation between NMs and plants, many studies indicate conflicting results: negative effects correspond to slower growth (Colman et al., 2013), necrosis (Lin and Xing, 2008), oxidative stress (Dimkpa et al., 2013), reduced photosynthetic activity (Wang et al., 2016; Dewez and Oukarroum, 2012; Barhoumi et al.,2014) and metabolic alterations. The same NMs could have positive effects (Miralles et al., 2012), for example improving germination, root elongation and seedling growth. In general, the response on plants depends on the type of NMs, particle size, treatment concentration, aggregation state, exposure time, environmental state and plant species (Liu et al., 2009; Schultz et al., 2014; Mrakovcic et al., 2013). It is already clear that aquatic and terrestrial organisms have been exposed to NMs, with a possible negative impact on human and ecosystem health, but scientific literature confirms that it is quite unknown the real kind of physico – chemical and biological effects and the ways of impact / interaction between NMs and environmental factors and living organisms in the long term (Cassee et al., 2011). For this reason, there is little information about the effects and the flows of these NMs in terrestrial and aquatic ecosystems (Simonin and Richaume, 2015; Eisenberg et al., 2015; Adam et al.,

2015; Dale et al., 2015; Dumont et al., 2015). Another important aspect is that many countries do not have a legal regulation for NMs and about their potential environmental impacts (FAO / WHO, 2013; Jain et al., 2016). For this reason, the increasing production of NMs, their release in the environment and the possible effects on ecosystem are concerns that need to be faced, especially by regulatory environmental agencies.

1.6 Nanoparticles and plants

Global biomass in planet Earth is dominated by plants, that are the primary producers in terrestrial and water ecosystems, which represent about 80% of the biota (Bar-On et al., 2018). Their life cycle is strongly dependent on their relationships with air, soil and water, and although for that reason they constitute the first biological target of engineered nanomaterials (ENMs), they are not considered among the environmental targets of ENMs by the Organization for Economic Cooperation and Development (OECD) guidelines (Hund – Rinke et al., 2016). It would be advisable to evaluate the impact of ENMs and consider the consequences in relation to the ecosystem services that plants provide (Isbell et al., 2011). Currently tons of ENMs such as TiO_2, CeO_2, FeO_x, AlO_x and ZnO are released in the environment (Montimer and Holden., 2019). ENMs flow models suggested that soils and waters are the end point of such materials (Keller et al., 2013; Holden et al., 2016). So, the other side of nanotechnology concerns the still lacking knowledge on the impact of these materials on biota (Reddy Pullagurala et al., 2019). Plants represent about 82% of biosphere (Bar – On et al., 2018). Studying the behaviour and fate of ENMs in plants is of extremely important for exploring ENMs uptake, translocation and storage in plant tissues; mechanisms of plant toxicity; life cycle risk

assessment of ENMs and risks of transfer them to the trophic chain. The early experimental demonstration regarding the negative influence of ENMs in higher plants was carried out not many years ago (Priester et al., 2012). Subsequent studies reported physiological and morphological anomalies of plant exposed to nanomaterials (NMs) (Miralles et al., 2012; Zhang et al., 2015; Zuverza Mena et al., 2017). However, scientific researches highlight several evidences of positive effects of ENMs applications to crops. That is why applications of nano products in crop nutrition and protection are under investigation (Duhan et al., 2017; Lowry et al., 2017; Kah et al., 2019). However, despite the obtained knowledge we still lack a systematic view regarding the effects of ENMs on plants and in particular on vascular plants. Until now, the research has almost exclusively concerned food crops (Ma and Yan, 2018; Sun et al., 2019), while little attention was devoted to spontaneous species. Although this was largely justified by the potential risks for human exposure to ENMs, potential negative impact of ENMs on primary producers could have very serious consequences on food chain and ecosystem services (Hawthorne et al., 2014) and therefore should not be considered less important. The studies carried out on crops, demonstrated that the ENMs impact on plants are influenced by the chemical and physical properties of NMs (e.g. size, shape, structure, concentration and others), the environmental conditions and the plant species (Duet et al., 2018; Spielman et al., 2019). Therefore, it is not advisable to generalize the results on crops (figure 3) to other plants living on natural ecosystems, neither fertilized nor irrigated and potentially more exposed to ENMs fluxes having longer life – cycle than crops. However, we lack studies on the relations between ENMs and no – food terrestrial plant species. Several, relevant papers concerning *Arabidopsis thaliana* (L.) Heynh. were published (Montes et al., 2017; Zhang et al., 2019; Milewska - Hendel et al.,

2019). Although it is a very useful model species for genomic and metabolomics studies, *A. thaliana* is evaluated as representative from the ecological point of view. Therefore, the number of spontaneous species studied is very low, according to studies on environmental ENMs fluxes in aquatic and terrestrial environments. However, more aquatic species (Asztemborska et al., 2018; Movafeghi et al., 2018; Ekperusia et al., 2019; Geitner et al., 2018) and wetland species (Yin et al., 2012); Jacob et al., 2013; Song et al., 2016) have been studied than terrestrial ones. According to Willis et al., (2017) the number of species of vascular plants currently known are about 391.000. Only about 150 species have a significant commercial value, and 20% of them account for more than half of the plants eaten by humans (Wiersema and Leon, 1999; Janick, 1999). Therefore, we optimistically assume that the relationships between ENMs and vascular plants have been studied on largely less than 0.05% of higher plants species. Again with regard to the issue of ENMs in terrestrial ecosystems, to the best of our knowledge, *Pinus sylvestris* and *Quercus robur* are the only no – food terrestrial plant species investigated for the exposure to ENMs (respectively of *n*Ag and *n*CeO_2; Aleksandrowicz – Trzcinska et al., 2019). Effective gap-filling actions are expected on this issue in the next future.

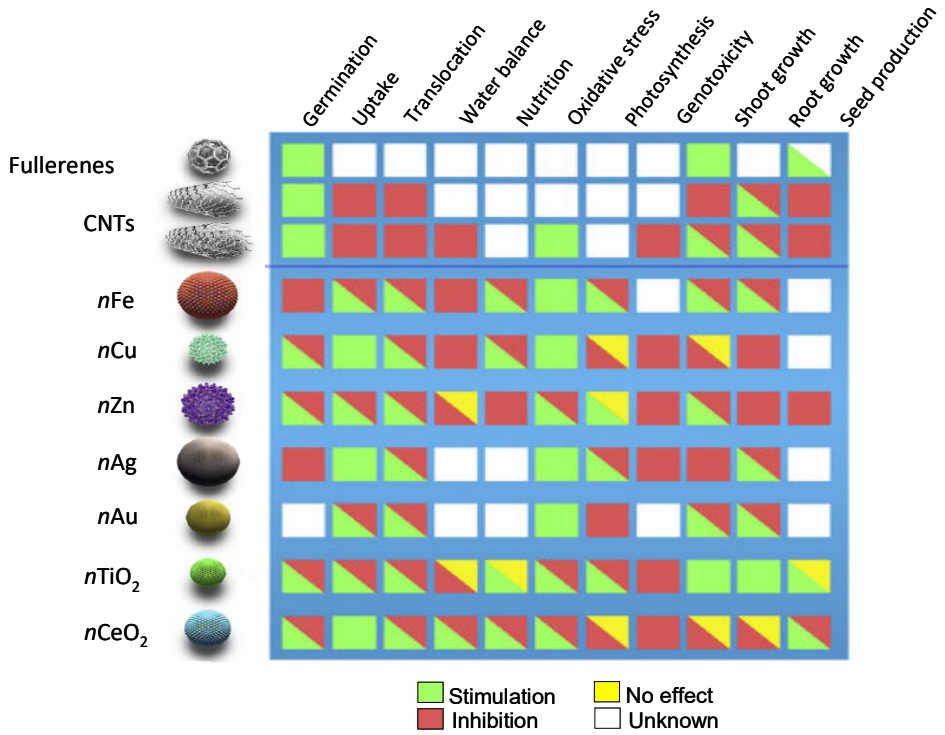


Figure 3: General trends of effects of engineered nanomaterials (ENMs) in crops. The matrix has been created using the plant responses under ENMs exposure. Contradictory responses have been detected (two colors in the same box), denoting that their effects depends on the specific kind of ENM, their concentration and the plant species (Zuverza – Mena et al., 2017).

1.7 Cerium oxide nanoparticles in plants

The current study evaluates the influence of *n*CeO_2 with different particle size and concentration not only on the early stage but also on the life-cycle of wild species. Nano ceria is considered because it is comprised among the top 10 most produced and widely utilized metal oxide nanoparticles in Europe (Piccinno et al., 2012; Meesters et al. 2016; Keller and Lazareva, 2014). They are used in the automotive industry as a catalyst or in electronic parts. The

rapid increase in the use of *n*CeO_2 nanoparticles in many application areas causes the spread of these materials in different environmental matrices and ecosystems. The Organization for Economic Cooperation and Development (OECD) included *n*CeO_2 in the list of engineered nanomaterials (ENMs) for immediate priority testing. Considering the soil matrix, *n*CeO_2 could go down into deeper layers after atmospheric precipitation or they could be absorbed by plants and in this way, they could spread along the trophic chain (Lopez - Moreno et al., 2010a; Hernandez - Viezcas et al., 2013). Several studies have shown that *n*CeO_2 have the potential to alter the physiology and biochemical processes of plants. Literature reports contradictory results regarding the relationships between *n*CeO_2 and plants. Regarding this topic, before starting the experimental activity described in this dissertation, it was studied and examined the theme of the effects of *n*CeO_2 on crops and most of the aspects were described in the following concentric overview: Lizzi D., Mattiello A., Marchiol L., 2017. *Impacts of cerium oxide nanoparticles (*n*CeO_2) on crop plants: a concentric overview*. Nanomaterials in plants, algae and microorganisms, Elsevier Book, Volume 1, Chapter 14.

1.8 Influence of *n*CeO_2 on plant growth

The remarkable and rapid increase in the use of *n*CeO_2 nanoparticles in many application areas caused the spread of these nanoparticles (NPs) even in the different environmental matrices. When *n*CeO_2 reached the soil, it is absorbed by vegetation and it could penetrate deeper into the soil layers, contaminating aquatic groundwater or it could also enter the food chain (Lopez - Moreno et al., 2010a; Hernandez - Viezcas et al., 2013). NPs generally come into plants

through root uptake or gaseous exchange of leaves. *n*CeO_2 could cause several effects on the plant system by reducing germination rates and improving, reducing or inhibiting radical growth (Lopez - Moreno et al., 2010a), where plants grow more slowly and reduce their leaf coverage or produce less biomass However, it is also observed that these NPs sometimes do not affect plant physiology and biochemistry (Ma et al., 2010). Responses given by plants depend on parameters such as plant species and particle size, treatment and concentration of *n*CeO_2. This chapter mainly discusses the effects of *n*CeO_2 exposure on edible plants in synchrony with studies already published. Studies on germination and seedling stage are examined and precede those on adult plants.

1.8.1 Effect on germination and root elongation

The exponential growth of nanotechnologies and its introduction to agriculture has prompted researchers to clarify the relationships between nanomaterials (NMs) and plants. During early stages of plant development, various parameters can be studied to evaluate the effects of exposition to toxic agents or other abiotic stresses. When germinating seeds are exposed to nanoparticles (NPs), different effects could be verified, depending upon species, particle size and concentration (figure 4) (Miralles et al., 2012). Different studies have reported different effects of *n*CeO_2 on crops.

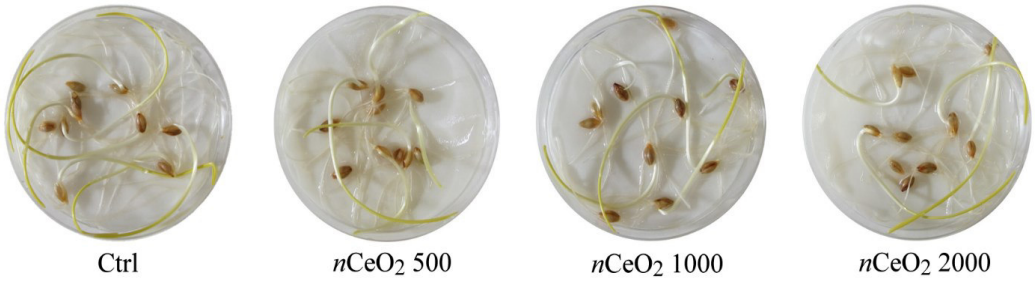


Figure 4 – Example of germination trial of *Hordeum vulgare* L. at different concentrations (0 - 2000 mg L^{-1}) of *n*CeO_2 (Mattiello et al., 2015).

López - Moreno et al. (2010a) observed the germination and root elongation of *Medicago sativa* L. (alfalfa), *Zea mays* L. (corn), *Cucumis sativus* L. (cucumber) and *Lycopersicum esculentum* L. (tomato). Both parameters were influenced by *n*CeO_2. In particular, a significant reduction in germination was recorded in tomato and corn at moderate concentrations. However, low toxicity was reported for alfalfa and cucumber, wherein contrasting evidence was recorded for root growth as well. At an *n*CeO_2 concentration of 2000 mg L^{-1}, root elongation was stimulated in cucumber and delayed in alfalfa and tomato. In the framework of a similarly organized experiment, Lopez - Moreno et al. (2010b) also studied the influence of cubic *n*CeO_2 on seed germination and early growth of soybean plantlets. No statistically significant effects on germination were reported, with the exception of the highest treatment (2000 mg L^{-1}). However, root growth increased by 75% in treated plants compared to control. In addition to this study, certain groups observed the potential of *n*CeO_2 genotoxicity. The random amplified polymorphic DNA (RAPD) technique was applied to root cell samples to verify the presence of genetic aberrations following exposure to *n*CeO_2. The results confirmed that higher concentrations of *n*CeO_2 are able to induce genotoxic effects on soybean plants (Lopez – Moreno et al., 2010b). Seed germination is commonly used as an endpoint in

phytotoxicity studies. Andersen et al. (2016) report the appropriateness of this parameter to screen *n*CeO_2 toxicity and the same was evaluated on some important crops. In this study, seeds of *Allium cepa* L. (onion), *Avena sativa* L. (oat), *Brassica oleracea capitata* L. (cabbage), *C. sativus*, *Daucus carota* L. (carrot), *Glycine max* L. (soybean), *Lactuca sativa* L. (lettuce), *Lolium perenne* L. (perennial ryegrass), *L. esculentum* and *Z. mays* were exposed to 0 - 1000 μg mL^{-1} of *n*CeO_2. Germination and early plant development were observed. According to Lopez - Moreno et al. (2010a; 2010b), it was verified that *n*CeO_2 does not cause acute toxicity in the early stages of plant development. Moreover, root elongation was more sensitive than germination. It is likely that the coated seed structures were able to protect seed germination, whereas the *n*CeO_2 exposure of seedlings determined different consequences. In fact, root elongation was stimulated in onion and ryegrass and stunted in lettuce and tomato. A further confirmation of the fact that seed germination is not influenced by the presence of *n*CeO_2 in the culture medium was provided by Mattiello et al. (2015). In their experimental conditions, the germination of *Hordeum vulgare* L. (barley) was not affected by *n*CeO_2 even at the highest level (2000 mg L^{-1}). Once more, the effects of the treatments were recorded at seedling stage. In particular, in this case seedlings exposed to the milder *n*CeO_2 concentration showed a reduction in root elongation compared to control.

1.8.2 Adult plant studies

To determine severity of effect potential interactions of *n*CeO_2 and crops were investigated not only in early stages of development but also over the entire

life cycle. The first aspect investigated by the researchers was the plant ability to take up *n*CeO_2. Then, several others traits of plant physiology and metabolism were studied. The accumulation of *n*CeO_2 in plants was firstly documented by Priester et al. (2012) in *Glycine max* L. cerium concentrations in the root tissues was linearly correlated with soil *n*CeO_2 concentration and accumulated into the roots with uptake levels similar to those observed by López-Moreno et al. (2010b). Also, the paper reported an evidence of great concern from an ecological point of view. Observations made by environmental scanning electron microscope (ESEM) showed that root nodules also acquire *n*CeO_2. Low levels of *n*CeO_2 did not significantly alter nitrogen fixation, however plant growth was stunted. With medium and high *n*CeO_2 concentrations, nitrogen fixation was severely affected. As regards, crop production treated plants had a lower number of leaves compared with control ones. In addition, crop yield was affected, as well. Although the number of pods per plant and seeds per pod were not affected by the treatment, a statistically significant reduction in the pod dry weight was recorded. Also Hernandez-Viezcas et al. (2013) investigated the uptake of *n*CeO_2 of soybean plants. In this case the Ce concentration in roots of treated plants resulted higher than those in control ones. However, the results demonstrated a low root-to-shoot translocation rate of the nCeO_2 in the aerial plant biomass. As for the possibility of direct uptake of *n*CeO_2, they investigated the Ce oxidation state in the plant tissues. The results demonstrate that about 79% and 88% of Ce detected in respectively root nodules and pods, was present as *n*CeO_2. Little is known about the physiological and biochemical responses of plants exposed to surface modified nanomaterials. On the other hand, continuous increments in the applications of coated *n*CeO_2 increase the chances for their dissemination in the environment, which could result in unpredicted effects on crop plants.

Barrios et al. (2015) compared the effects of uncoated *n*CeO_2 and citric acid coated *n*CeO_2 on the growth, fruit production, uptake of Ce and essential elements, as well as chlorophyll content in *Lycopersicon esculentum* o Solanum lycopersicum L. (tomato). The activity of catalase (CAT) and ascorbate peroxidise (APOX) enzymes were measured as well on the fully developed plants. The treatments with coated and uncoated *n*CeO_2 did not affect the nutrient element uptake and plant metabolism. The release of ionic Ce was smaller in coated Ce than in uncoated. As a consequence, a lower Ce root uptake was observed. Tomato plants were used as model plant by Vittori Antisari et al. (2015) which studied the influence of *n*CeO_2, *n*Fe_3O_4, *n*SnO_2, *n*TiO_2) and metallic *n*Ag, *n*Co, *n*Ni on plant growth, the possible uptake of nanoparticles and the content of nutrients (Ca, Mg, K, Na, P and S) in different tomato organs. *n*CeO_2 treatment did not affect stem and root elongation, whereas a decrease in the dry plant biomass was recorded. Considering the root accumulation of macronutrients, *n*CeO_2 (with nCo and *n*Fe_3O_4) are able to inhibit K translocation to stem and leaves. Moreover, *n*CeO_2 does not allow Ca, Mg and P to be translocated to leaves. According to Carvalho Bertoli et al. (2012), this evidence suggests a competitive inhibition between K and other cations, e.g. metal component of nanomaterials. Cerium did not translocate in the treated tomato, perhaps due to the size of the *n*CeO_2 employed in the experiment (50-105 nm). Since the potentially different impact of *n*CeO_2 and their bulk counterparts to plants is not so clear, Ma et al. (2016) studied the physiological and biochemical adjustments at different growth stages in *Brassica rapa* L. The results indicated that bulk CeO_2 enhanced plant biomass while *n*CeO_2 did not. While the bulk CeO_2 treatment resulted in significantly higher hydrogen peroxide (H_2O_2) in plant tissues at the vegetative stage, *n*CeO_2 led to significantly higher H_2O_2 in plant tissues at the floral stage. SOD-activity

had a growth-stage dependent response to different sizes of *n*CeO_2. CAT-activity was not affected by either size of *n*CeO_2 throughout the life cycle of *Brassica rapa*. Finally, we consider the most recent papers by Zhang et al. (2017) and Servin et al. (2017). Zhang et al. (2017) studied the *n*CeO_2 uptake by sand cultured *Lactuca sativa* L. (romaine lettuce). Also in this case, it was demonstrated the dose - dependent accumulation of Ce in plant roots. Moreover, transmission electron microscopy (TEM) observations confirmed the presence of *n*CeO_2 aggregates both in the root surface and within root tissues (in the intercellular space) and within root cells (vacuole). Once confirmed the relationships between *n*CeO_2 and plant uptake, the authors studied the potential effects on plant metabolism and growth. In particular, the superoxide dismutase (SOD), peroxidase (POD) activities and malondialdehyde (MDA) contents were analyzed in roots and shoots tissues. At higher concentrations (1000-2000 mg kg^{-1}) *n*CeO_2 caused a decrease in chlorophyll content and in fresh and dry weight of plant fractions. The present study suggests *n*CeO_2 could induce significant oxidative damages, reduction of chlorophyll content, and decrease of biomass production to romaine lettuce. Moreover, romaine lettuce showed higher sensitivity to *n*CeO_2 in sand media than that in soil media. These results indicate that plants increased the production of antioxidant enzyme activities to defend the oxidative stress. *n*CeO_2 at high concentrations induced higher oxidative stresses which can't be effectively attenuated by the antioxidant system. According to Rico et al. (2013b) these results confirmed that oxidative stress was involved in the toxicity of *n*CeO_2 to romaine lettuce.

The knowledge regarding the interactions between engineered nanomaterials and soil are still limited. However, soils are known to contain or be amended with organic matter, humic substances and biochar that can significantly interact with nanomaterials. In particular, some experimental evidences by Yi

et al. (2015) showed that biochar and *n*CeO_2 tend to aggregate due to the attraction of negatively charged sites on biochar and the positively charged surfaces of *n*CeO_2. In connection with this, Servin et al. (2017) studied the effect of interactions between *n*CeO_2 and biochar on Ce accumulation, biomass, chlorophyll production and lipid peroxidation in *Cucurbita pepo*, *Glycine max*, *Lactuca sativa* and *Zea mays*. The plants were grown in two different soils amended with two different types of biochar. The results showed that the biochar type influenced significantly the interactions between *n*CeO_2 and plants, with relevant consequences on particle fate.

2. AIM OF THE STUDY

The aim of this research is to illustrate how nanoparticles (NPs) may affect plants and the possible effects that NPs could have on plants life cycle. In scientific literature, different experiments, studies and reviews examine the interaction between nanomaterials (NMs) and crops and cultivated species, and in particular about the effects that these new materials have on their life cycle. The experiments described consider three common and spontaneous plant species, which are widespread in natural systems and easily adaptable to different ecological conditions. In particular, the effects of NPs on spontaneous plant species are investigated using different types of NPs but with the same class size. In this study, it has been decided to use only one NM but with different particle dimensions. The main innovation of this research is the use of cerium (Ce), which is one of the most produced and widely utilized NM in

Europe, with two different particle class size, specifically 25 nm and 50 nm. Therefore, the goals of this work were to:

- determine the effects of *n*CeO_2 on spontaneous plant species in early plant development stages and during the entire life cycle;
- quantify the capacity to uptake and the possible translocation of Ce through the plant tissues, from roots to stems and leaves;
- examine the effects of different concentrations and different size of *n*CeO_2 in order to define a possible influence related to the particle dimensions;
- highlight possible signals of bioaccumulation of cerium in plant tissues after repeated treatments.

The results of this study could be useful to improve not only the knowledge about the interactions between NMs and plants but more in general with the environment, because the behavior, the fate and in particular the effects of these materials on spontaneous plant species and natural matrices are still quite completely unknown. This is why, similar studies could be able to reveal important ecological concepts and information for future ecological risk assessment efforts, in particular where there are no laws that norm the presence of these new materials in the environment, which could become the new contaminants in the future.

3. MATERIALS AND METHODS

3.1 Plant species

In the experiments that will be described, three common and spontaneous plant species are considered: these are *Holcus lanatus* L., *Diplotaxis tenuifolia* (L.) DC. and *Silene flos cuculi* L. These species are fast growing plants and produce biomass in very little time; they are also been choose because of having a general view on plant taxonomic groups. Indeed, one of them is a monocotyledonous (*H. lanatus*) while the others are dicotyledonous.

Figure 5: *Holcus lanatus* L.

Holcus lanatus L. (common velvet grass) in figure 5 is a hairy, tufted, fibrous-rooted and meadow soft perennial grass, growing between 50 an 100 cm tall, belonging to *Poaceae* family (Stace, 1997). In North America, this plant is an invasive species in grasslands and other ecosystems, because it forms dense

stands that could exclude other plants. Common native species in Northern Europe, *H. lanatus* has a wide climatic range and is commonly found on pasture, roadsides, waste ground, open woodland and riverbanks. It occurs over a wide range of soil types and fertility conditions (Thompson and Thurkington, 1988). This species has long green leaves, the ligule is around 4 mm long and the stems present pink veins at the bases; it produces a large amount of seeds that could rapidly colonize disturbed grounds. *H. lanatus* is characterized by the absence of rhizomes and it spreads by developing new shoots and roots at its nodes and forming a blanket of runners on the soil surface.

Figure 6: *Diplotaxis tenuifolia* (L.) DC.

Another plant species observed in this study was *Diplotaxis tenuifolia* (L.) DC. (Perennial wall rocket) in figure 6: it is a Brassica perennial flowering

herbaceous plant and a Mediterranean species but it is native to Europe and Western Asia (Hall et al., 2012b). For this reason, it grows in temperate world, where it is naturalized from sea level to 1400 m a.s.l. It could be found in different habitats but in particular in ruderal plant associations, along roads, in landfills, on cliffs and walls, in abandoned fields and ruderal areas, more rarely in cultivated fields. *D. tenuifolia* grows in clumbs and it is an erect plant with branching stems that could reach half a meter in height; this species has long lobed aromatic leaves and at the top of the branches, it develops yellow flowers with rounded petals. It produces straight siliques which contain small brown seeds.

Figure 7: *Silene flos – cuculi L*.

Silene flos - cuculi L., synonym of *Lychnis flos - cuculi* L. (ragged – robin) in figure 7 is a herbaceous perennial wetland plant, belonging to *Caryophyllaceae* family. This species is native and distributed throughout Europe (Jalas and

Suominen, 1986) where it is found in moist open habitats, along roads and in wet meadows and pastures, but it also grows in the Northern United States and Eastern Canada. S. flos-cuculi forms vegetative rosettes with numerous flower stems that could be from 20 to 90 cm tall. The stems have barbed hairs that make the plant rough to the touch; stems grow over the foliage and end with pink flowers, which open between April and June and many types of insects are attracted by flowers nectar. Another characteristic of these flowers is that they have 5 narrow petals divided into four lobes. The leaves are paired: the lower ones are stalked and the upper leaves present pointed apexes. The fruits consist in small capsules, containing many dark seeds, that could be dispersed mechanically (Biere, 1991).

3.2 Nanoparticles characterization

Different studies in literature highlight the effects of nanomaterials (NMs) on plants and in particular on agricultural and cultivated species, focusing the attention on stimulation / inhibition of germination, root elongation, plant development and consequently seed production. Moreover, in most of these studies, researchers use different nanoparticles (NPs) but with the same class size. In the present study, it has been decided to use one NM with 2 class size because size and shape are the most important parameters that influence the behaviour of engineered nanomaterials (ENMs). For the experiments, we used cerium, which is one of the most widely used ENMs in the world. NPs characterization was carried out at the laboratories of National Research Council – Institute of Science and Technology for Ceramics (Faenza, Italy). The

cerium oxide nanopowders (nCeO$_2$) with an average particle size of 25 nm and 50 nm respectively, were purchased from Sigma – Aldrich (St. Louis, MO, USA). These nCeO$_2$ have MW of 172.11 g mol^{-1} and density of 7.13 g mL^{-1} at 25°C. nCeO$_2$ have 99.95% of purity (81,25% of Ce). The nCeO$_2$ are suspended in deionized water and sonicated in a water bath for 60 minutes with a sonication intensity of 180 watts. The nCeO$_2$ powder suspensions at 2000 mg L^{-1} were prepared in MilliQ water by sonication for 30 minutes and then stirred for 15 minutes. The suspensions were characterised for Z – average size, zeta potentials and hydrodynamic diameter (Hd), which distributions were measured by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Ltd., Worcestershire, UK) (figure 8). Zeta – potentials were quantified by laser Doppler velocimetry as the electrophoretic mobility, using a disposable electrophoretic cell (DTS1061, Malvern Ltd., Worcestershire, UK).

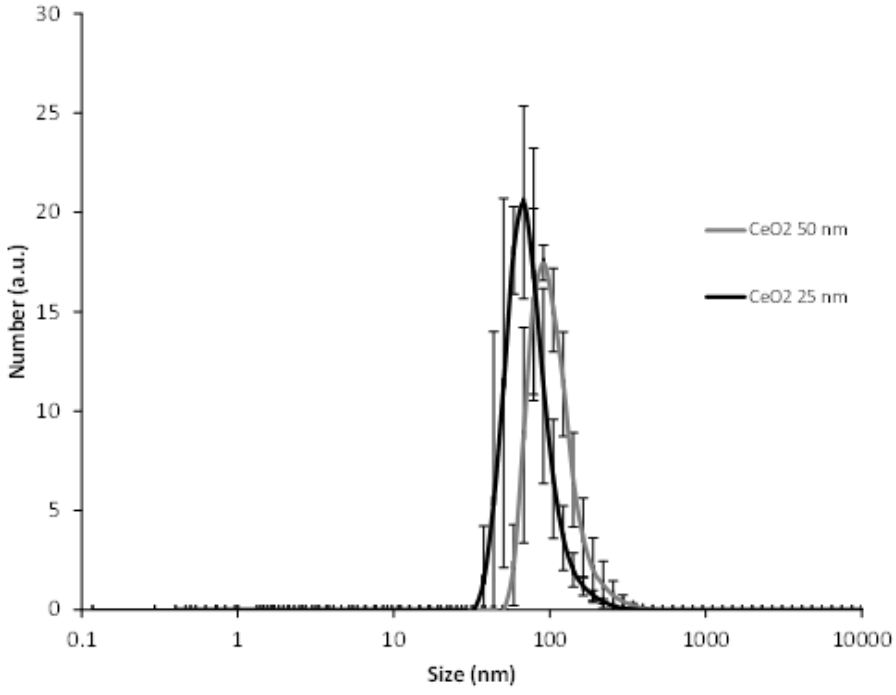


Figure 8: Characterization of nCeO$_2$ (25 and 50 nm), obtained by dynamic light scattering (DLS).

Table 2: Z- average, PDI and ζ-potentials of nCeO2 (25 nm and 50 nm).

Material	Z - average (nm)	PDI	ζ - potential (mV)
nCeO_2 25 nm	126.7±1.0	0.17±0.01	39.2±1.1
nCeO_2 50 nm	205.7±1.0	0.25±0.02	24.1±0.8

An aliquot of each sample was suspended in Arium ultrapure water to reach a concentration of 0.5 mg mL^{-1} and sonicated in a bath sonicator for 10 minutes at 25°C. The relative Z – averages are reported in table 2 together with the relative polydispersity index (PDI) and the ζ – potentials of the particles. Both the samples exhibit a monodisperse size particle distribution in the nanometric range with relatively low PDI, and a main size peak at 91 nm and 62 nm, respectively. The Z - averages calculated for both the samples are much larger than these values, as this parameter takes into account the size of larger particles aggregates. The ζ - potentials measurements revealed that *n*CeO_2 25 nm has a much higher net surface charge with respect to *n*CeO_2 50 nm. As high net surface charge is typical of nanoparticles suspension with minor aggregation, these data are coherent with the higher aggregation found for sample *n*CeO_2 50 nm, as denoted by its higher Z - average with respect to *n*CeO_2 25 nm. The size and average shape were measured with transmission electron microscope (TEM, FEI Tecnai F20): figure 9 A) describes 25 nm and figure 9 B) is about 50 nm.

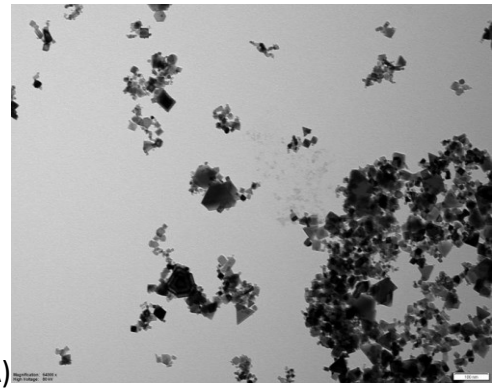
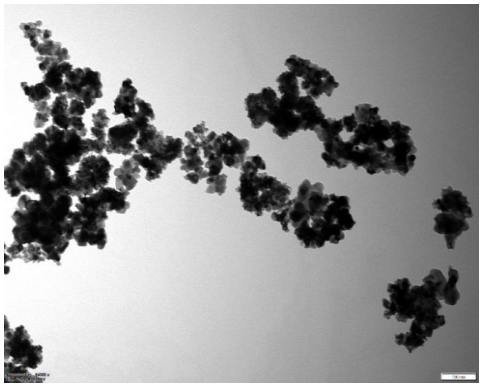
A)  B) 

Figure 9: TEM images of *n*CeO_2 A) 25 nm and B) 50 nm.

3.3 Internalization of *n*CeO_2 in plant tissues

Before starting the experiments, a preliminary study was done to verify the uptake of *n*CeO_2 by seedlings and with an enzymatic digestion, the entry of nanoparticles (NPs) in plants was effectively verified. Seeds of *D. tenuifolia*, *H. lanatus* and *S. flos cuculi* were sown into Petri dishes and watered with *n*CeO_2 suspensions at concentration of 2, 10, 20, 50, 200 and 2000 mg L^{-1}. Cultivation carried on for 10 days after the germination, to obtain seedlings at the cotyledon stage. After cultivation, the small plants were harvested, washed with MilliQ water and 0.03 g of fresh plant samples were homogenized with 8 mL of 2 mM citrate buffer at pH 4.5, using an ultrasonic bath for 5 minutes. We used this pH because it is inside the optimum application range of the applied digesting enzyme: Macerozyme R – 10 enzime – pectinase from *Rhizopus* sp. (Sigma Aldrich); so the pH of citrate buffer was adjusted with citric acid till arriving to a value of 4.5. The extraction of *n*CeO_2 from homogenized samples of these species was performed according to Jimenez – Lamana et al., (2016); the whole scheme of analytical procedure for the enzymatic digestion of plants

and for samples preparation is presented in figure X. After the homogenization, for every sample 2 mL of the enzyme solution (0.05 g of enzyme powder for roots, shoots, leaves and seedlings, dissolved in 2 ml of MilliQ water) were added. The samples were incubated and shaken in a water bath at 37°C for 24 h. After the incubation, all the obtained suspensions were filtered with a 0.45 µm cellulose filters to remove the solid parts of seedlings, which remain after homogenization and incubation. The final supernatant of *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* was appropriately diluted 10 times using ultrapure water before the analysis by SP - ICP – MS. All the samples were analysed using NexION 350 ICP – MS (PerkinElmer) (figure 10), operated in the single particle mode, using Single Nanoparticle Application software (Syngistix Nano Application Module).

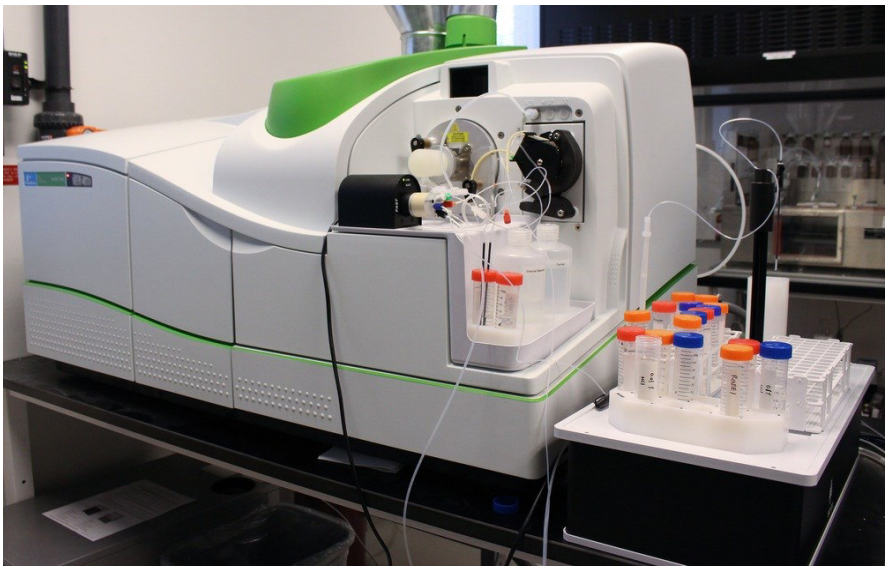


Figure 10: NexION 350 ICP – MS (PerkinElmer) that analyses samples to obtain total content of Ce and *n*CeO_2 (using Syngistix Nanoparticle Application Module).

3.4 Seed germination and root elongation

After the preliminary test conducted for all species and described in the previous paragraph, it was possible to proceed with the complete experiment, which had the objective of assessing the germination and root length of seeds planted in Petri dishes, treated at different concentrations and with different sizes of cerium oxide nanoparticles (*n*CeO_2). Seeds of *D. tenuifolia* were provided by Sementi Bruni (Corbetta, Milan, Italy) instead seeds of *H. lanatu*s and *S. flos cuculi* were purchased by SemeNostrum (Udine, Italy). 30 seeds were placed into 15 mm Petri dishes containing filter paper soaked with 10 ml of MilliQ water (control treatment) or 10 mL varying concentrations of *n*CeO_2 suspensions. The suspensions of *n*CeO_2 for the germination experiment were prepared at different concentrations (0, 2, 10, 20, 50, 200 and 2000 mg L^{-1}) in MilliQ and double class size (25 nm and 50 nm), stirred by 10 minutes to avoid aggregation and sonicated for 30 minutes. The concentrations were chosen according to Yang and Watts (2005), Lin and Xing (2007) and Lopez – Moreno et al., (2010a). The preliminary experiment was also necessary for knowing plant time of growing for each kind of species. Petri dishes were covered and they were wetted every 3 days with the corresponding treatment.

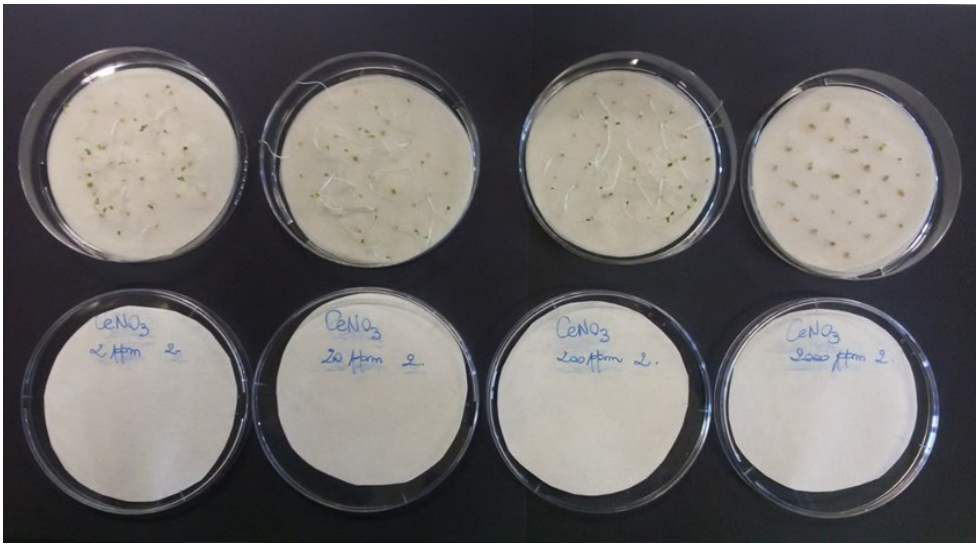


Figure 11: Seed of *D. tenuifolia* germinated in Petri dishes after 5 days; seeds are treated with *n*CeO_2 (25 nm and 50 nm) and $CeNO_3$ at different concentrations (0, 2, 10, 20, 50, 200 and 2000 mg L^{-1}).

The germination percentage was calculated as the ratio of germinated seeds out the total seeds of each Petri dish (30 seeds) (figure 11) after 2 weeks of incubation (Hou et al., 2014; Wang et al., 2016a; Wang et al., 2017c; Wang et al., 2017d). The seedlings were photographed (figure 12) and Image J software was used to measure roots length (Schneider et al., 2012). Root elongation was calculated as the average of measures of all roots emerged from each seed (for each Petri dish). The experiments were performed with 3 replicates for every treatment and the duration of the experiment was of 2 weeks.

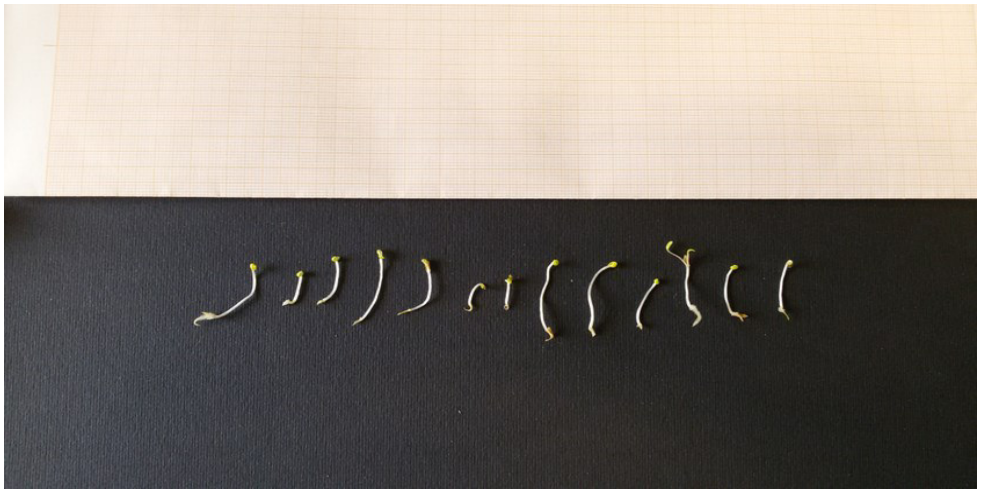


Figure 12: Seedlings of *D. tenuifolia* treated with *n*CeO_2 (25 nm and 50 nm) and $CeNO_3$ at different concentrations (0, 2, 10, 20, 50, 200 and 2000 mg L^{-1}), arranged to calculate root length.

3.5 Plant growth and life cycle exposed to *n*CeO_2

The second experiment was designed with the aims of observing the impact and the effects of prolonged exposure of plant species to *n*CeO_2 and verify the possible translocation of nanoparticles (NPs) in plant fractions. The experiment was conducted on plants raised in a microcosm and semi - controlled conditions (greenhouse). Also for this experiment, seeds of *D. tenuifolia* were provided by Sementi Bruni (Corbetta, Milan, Italy) instead seeds of *H. lanatus* and *S. flos cuculi* were purchased by SemeNostrum (Udine, Italy). The soil used for this experiment is COMPO SANA organic potting mix, a mixture of forest products, compost, perlite and wetting agent and fertilizer (soil pH = 6.8 - 7.2). The soil characterization is contained in table 3.

Table 3: Characterization of COMPO SANA organic potting mix.

B	Ca	Cu	Fe	K	Mg	Mn	Zn
89.9 ± 5.52	28099 ± 484	151 ± 11.8	12734 ± 130	8685 ± 35.8	3573 ± 14.7	351 ± 4.47	1307 ± 0.13

This experiment was divided in 2 sub experiments: in the first, *H.lanatus* and *D. tenuifolia* were exposed to *n*CeO_2 concentrations of 0, 2, 20 and 200 mg kg^{-1} and the class sizes were the same of the germination experiment (25 nm and 50 nm). The treatments were made by adding *n*CeO_2 solutions to the soil and mixing it to obtain the prearranged concentrations. Suspensions were prepared with MilliQ water; they were stirred and sonicated for 30 minutes to avoid aggregation before homogeneous mixing with the soil. The addition of NPs occurred in soil in a single dose before sowing through an irrigation. The soil was irrigated with the corresponding suspension / solution and the calculations were done according to the amount of potting soil used per pot (500 mL for 500 g of soil). The soil was left for 48 h for conditioning before planting seeds; after soil equilibration, 500 g of soil amended with *n*CeO_2 or control soil were put in each pot. In this way, plants grew for the entire life cycle in solid substrate enriched with *n*CeO_2 of different sizes (25 nm and 50 nm) and at different concentrations (2 - 20 - 200 ppm). For every plant species, the experiment was composed of 24 replicates, 4 for each Ce concentration and class size. In the second sub experiment, *S. flos cuculi* was exposed to repeated applications of *n*CeO_2, at concentrations of 0, 20 and 200 mg kg^{-1}. The *n*CeO_2 class size chosen for this experiment was 25 nm, because easily absorbed by plants. As for *H. lanatus* and *D. tenuifolia*, the treatments were made by adding *n*CeO_2 solutions to the soil. The first addition of NPs occurred in soil with one dose before sowing through an irrigation. The soil was irrigated with the

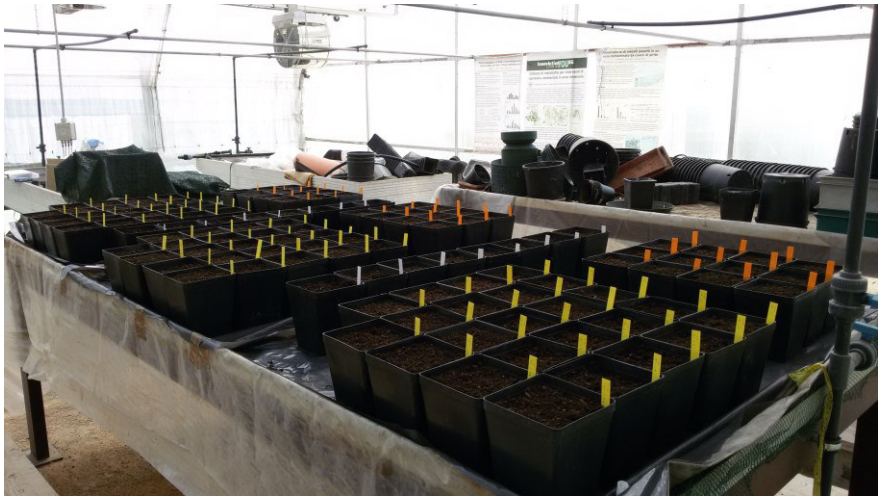
corresponding solution, according to the amount of potting soil used per pot (500 mL for 500 g of soil). Also in this sub experiment, the soil was left for 48 h for conditioning before planting seeds. Repeated applications of *n*CeO_2 were done respectively after 20 and 40 days from seedling emergence (figure 15). During the additional treatments, plants of *S. flos cuculi* were irrigated with solutions containing the same *n*CeO_2 concentrations (0, 20 and 200 mg kg^{-1}) of those used previously to amalgamate the substrate. In this way, it was tried to recreate a situation of chronic "contamination". In both experiments, seeds were planted in each pot, followed by the removal of small seedlings after germination, because only 4 plants per microcosm were cultivated to study the life cycle of species. The greenhouse experiment started at the experimental farm of the University of Udine (Italy) on the 30th of May 2018. The seeds of *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* were put about 2.5 cm deep in the soil and watered with water every day, using an automatic irrigator to ensure constant hydratation. Pots were placed in a greenhouse under full sunlight (figure 12), at around 18°C during the night and 27°C during the day, with the relative humidity over 60%. Control plants grew under exactly the same conditions, not exposed to NPs treatments for the entire period of the experiment.

A)

B)

Figure 12: Pot soil experiment in the greenhouse: in image A), topsoil amended with *n*CeO_2 solutions (25 and 50 nm) at different concentrations (0, 2, 20, 200 mg L^{-1}); in images B), topsoil put in pots and classified for treatments.

C)



D)

Figure 12: Pot soil experiment in the greenhouse: in image C), topsoil put in pots and classified for treatments: in image D), seedlings of *H. lanatus*, *D. tenuifolia* and *S. flos cuculi*.

Seed germination started after the third day for *D. tenuifolia* and after the fourth one for *H. lanatus* and *S. flos cuculi*. The pictures below (figure 13 A), B) and C) and figure 14 A), B) and C) describe the development and growth of plants during the experiment.

A)

B)

C)

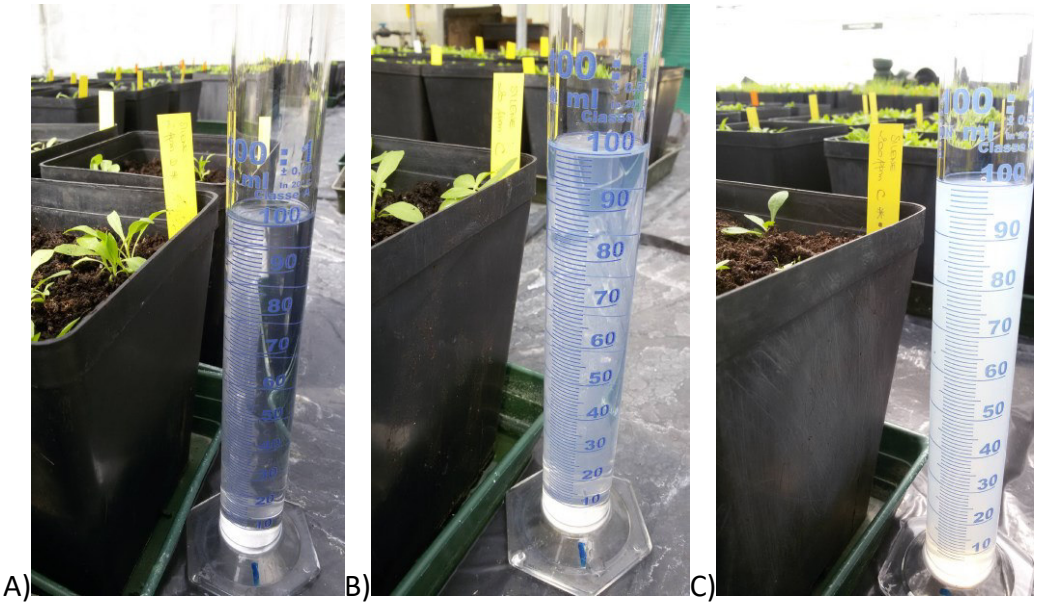
Figure 13: Pot soil experiment in the greenhouse: in images A), B) and C) plants of H. lanatus, D. tenuifolia that grow in topsoil amended with nCeO2 solutions (25 and 50 nm) at different concentrations (0, 2, 20, 200 mg L-1) during their life cycle.

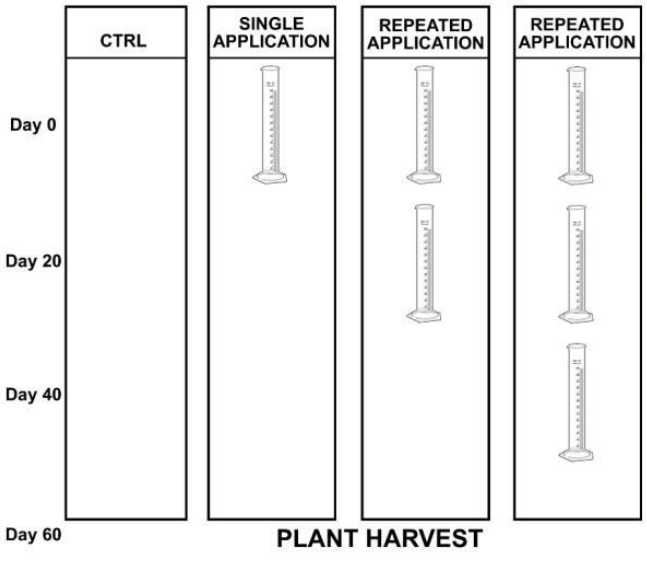
A)

B)

C)

Figure 14: Pot soil experiment in the greenhouse: in images A), B) and C) plants of *S. flos cuculi* that grow in topsoil amended with *n*CeO_2 solutions (25 nm) at different concentrations (0, 2, 20, 200 mg L^{-1}) during their life cycle.





D)

Figure 15: Additional treatment of nCeO$_2$ after 20 days and after 40 days (25) at different concentrations: A) corresponds to 2 mg L^{-1}; B) to 20 mg L^{-1} and C) to 200 mg L^{-1}; D) the summary scheme of treatments.

After 60 days, control and treated plants were harvested, washed individually with MilliQ water and separated to parts. In particular, the greenhouse experiment ended with the evaluation of the biometric parameters of the species (dry weights, leaf area, number of leaves, total dry matter). Prior to harvest the plants, their height was measured from soil surface to flag leaf, using a standard meter. Then, plant shoots were cut at the collar with a blade and then plants have been divided into different sections (roots, stem and leaves, as in figure 16 below) and weighed (fresh weight and dry weight after drying in oven at 60°C for 3 days).

Figure 16: Roots, stems and leaves of *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* plants, before laboratory analysis.

3.6 Total content of Cerium in plant tissues

To quantify the content of Ce inside different plant species, in the first experiment, seedlings were washed by agitation with HNO_3 0.01 M for 15 minutes and rinsed 3 times with MilliQ water. For the greenhouse experiment, plant sections were accurately washed with tap water and rinsed 3 times with MilliQ. Then both seedling and plant sections were oven - dried at 60°C for 3 days and 0.3 g of tissues were digested on a CEM microwave oven (MARS Xpress, CEM, Matthews, NC, USA), using 9 mL of HNO_3 and 1 mL of hydrogen peroxide (H_2O_2) in Teflon cylinders at 180°C, according to the USEPA 3052 method (USEPA, 1995). After digestion, all samples were transferred into 20 mL flasks (figure 17) and appropriately diluted with MilliQ water; plant extracts were filtered with Whatman 0.45 µm PTFE membrane filters. Determinations of the total content of Cerium was performed using NexION 350 inductively coupled plasma mass spectrometry (ICP – MS, Perkin Elmer, USA). Yttrium was the internal standard used for the analysis (Packer et al., 2007). In addition, a 0.05 mg L^{-1} Ce standard was analysed every 20 samples to control the correct readings of the instrument and the accuracy of the analytical procedure adopted.

Figure 17: Samples in 20 mL flasks, where plant samples are transferred after acid digestion and before filtration.

3.7 Data analysis

Both laboratory and greenhouse experiments were set up as a randomised factorial design of treatments and each week Petri dishes and pots were randomly reallocated. After the end of the experiments, the huge amount of obtained data were verified to determine the deviations from normality before data analysis. The differences in plant behaviour were determined by one-way and two-way analysis of variance (ANOVA). Statistically significant differences correspond to P equal to or less than 0.05. Tukey's Multiple Comparison test (p = 0.05) in case of significant effects were used to analyse individual effects. Different letters in tables and graphics are used to indicate means that are statistically different at p = 0.05. Statistical analysis is performed using CoStat Statistics Software.

4. RESULTS AND PRELIMINARY DISCUSSIONS

4.1 *n*CeO_2 extraction after enzymatic digestion of seedlings

In scientific literature, different studies and reviews are referred to the interaction between nanomaterials (NMs) and crops / cultivated species. In particular, they describe the effects that these new materials have on plants life cycle, focusing the attention on stimulation / inhibition of germination, root elongation, plant development and consequently seed production. For this reason and because of the lack of knowledge, three common and spontaneous plant species are studied: *H. Lanatus*, *D. tenuifolia* and *S. flos cuculi*. It is one of the first study that considers this type of plants in an experimental context with NMs. Moreover, in the present work, it has been decided to use only one NM, which is cerium (Ce): one of the most widely used engineered nanomaterials (ENMs) in the world, but with double class dimensions. This decision is justified by the fact that size and shape are two of the most important features that identify the NMs. The preliminary study was done to verify the uptake of *n*CeO_2 in seedlings after the enzymatic digestion of plant tissues with Macerozyme – R10. Samples at high concentration (50 and 200 mg L^{-1}) are diluted 100 times and the other samples (2, 10 and 20 mg L^{-1}) are diluted 10 times before single particle analysis (SP – ICP – MS). The results of the analysis of treated samples demonstrate that both class sizes of *n*CeO_2 (25 nm and 50 nm) are absorbed by roots and after this passage, they are transported in the other parts of the seedlings.

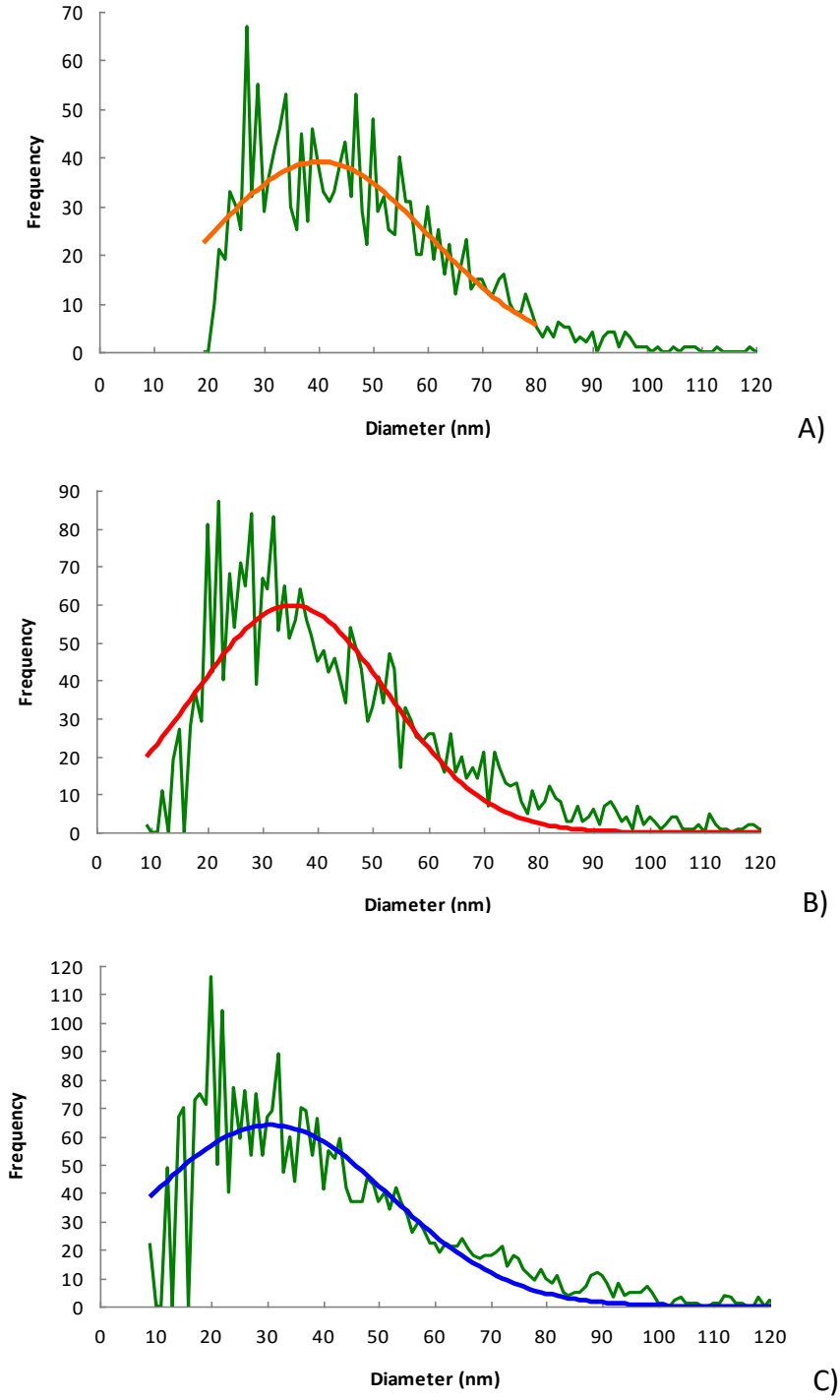


Figure 18: Size distribution obtained for nCeO$_2$ 25 nm at 20 mg L^{-1} in the three species after enzymatic digestion (A) orange line = *H. lanatus*; B) red line = *D. tenuifolia*; C) blu line = *S. flos cuculi*).

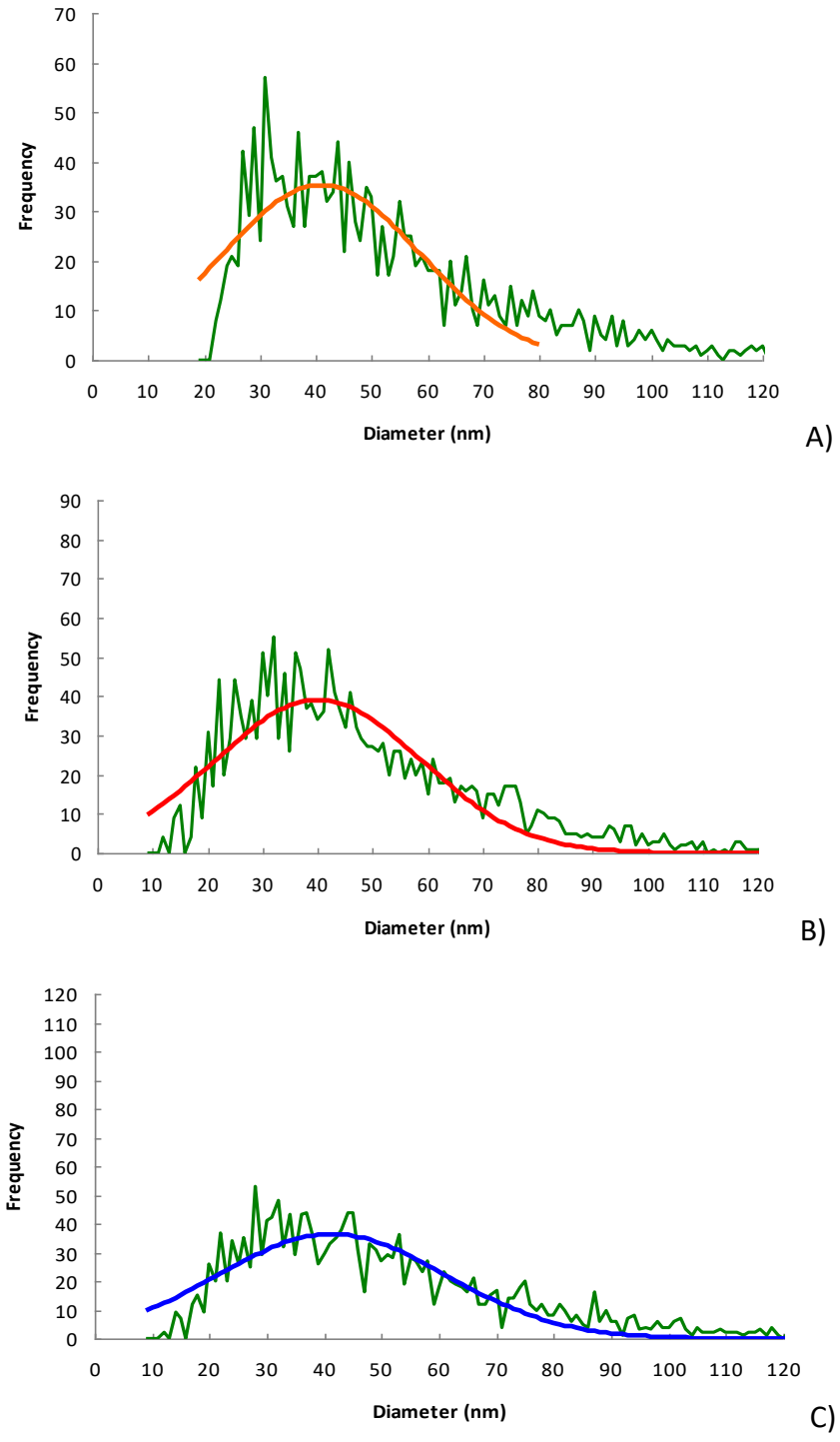


Figure 19: Size distribution obtained for *n*CeO_2 50 nm at 20 mg L^{-1} in the three species after enzymatic digestion (A)orange line = *H. lanatus*; B) red line = *D. tenuifolia*; C) blu line = *S. flos cuculi*).

Similar size distribution was obtained for 25 nm in all the species but in particular in dicotyledonous ones (*D. tenuifolia* and *S. flos cuculi*), which graphics are almost superimposable (figure 18 A), B) and C)). No significant changes in nanoparticles (NPs) size were observed for plants treated with 50 nm solutions (figure 19 A), B) and C)).

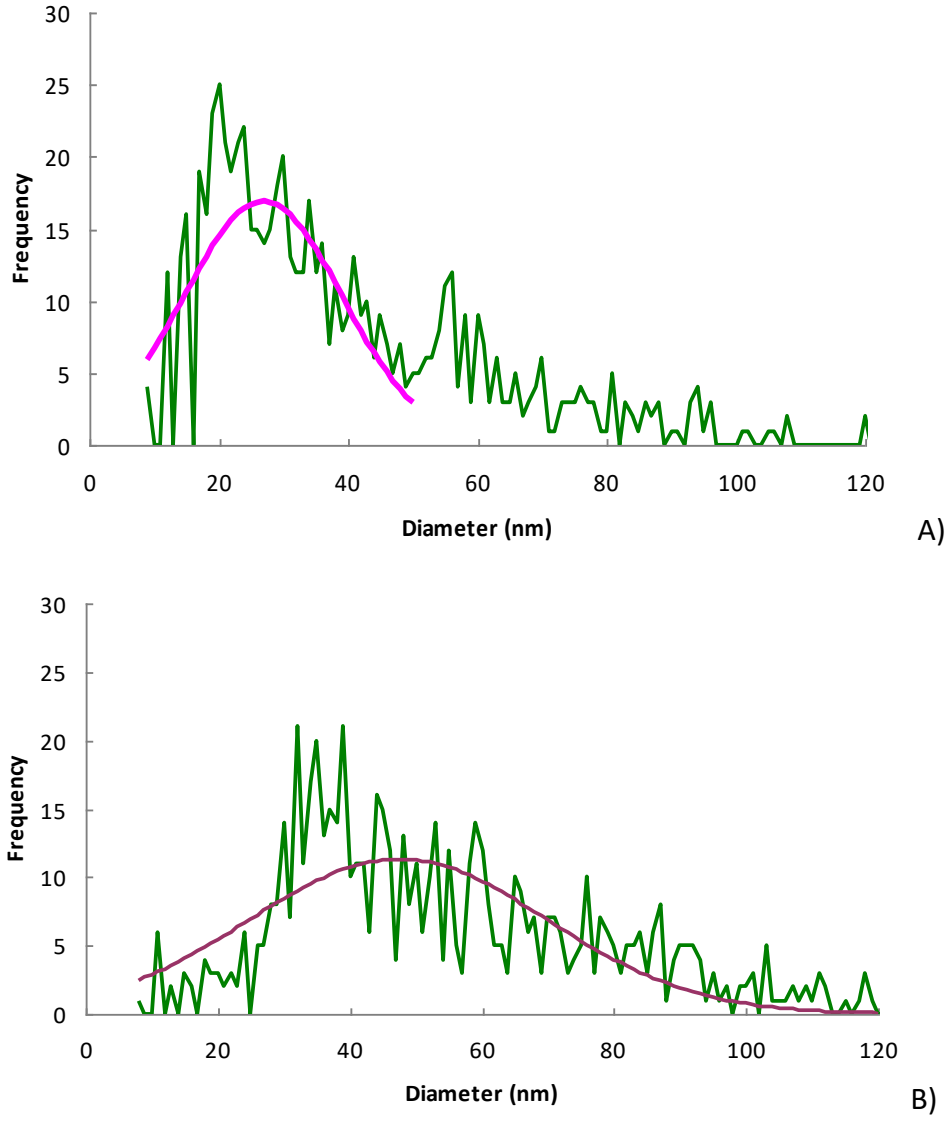


Figure 20: Size distribution obtained for stock suspensions of nCeO$_2$ at 20 mg L^{-1}: A) pink line = 25 nm; B) purple line = 50 nm.

The analysis gives the opportunity to observe a sort of agglomeration process of *n*CeO_2 in the seedlings of all the three species treated with 25 nm NPs, if compared with the graphics of the two *n*CeO_2 stock suspensions, used in this experiment (figure 20 A) and B)).The agglomeration verified in particular after *n*CeO_2 treatments (for example, mean size of 46, 43, 40, 41 and 45 nm in *H. lanatus*, respectively at 2, 10, 20, 50 and 200 mg L^{-1} of 25 nm solutions) could be justified with the attraction of NPs caused by van der Waals forces or chemical bonds. Similar results were obtained in *D. tenuifolia* and *S. flos cuculi*. The highest number of NPs observed at 50 and 200 mg L^{-1} could be due to a direct contact of roots with the solution used for seed germination in Petri dishes. Comparing the results of the three species, it is observed that the NPs concentration is higher for dicotyledonous species (*D. tenuifolia* and *S. flos cuculi*) than monocotyledonous (*H. lanatus*) and in particular for 25 nm treatments.

4.2 Seed germination and root elongation

After the preliminary test conducted for all species and described in the previous paragraph, it was possible to complete the experiment, with the objective of assessing the effects on germination and root length of seeds planted in Petri dishes, treated at different concentrations and with different sizes of cerium oxide nanoparticles (*n*CeO_2). At the end of the experiment, seeds produced a simple single root and two small leaves (coleoptile). The effects of *n*CeO_2 treatments on seed germination and root elongation of the three spontaneous species are shown in the following graphs.

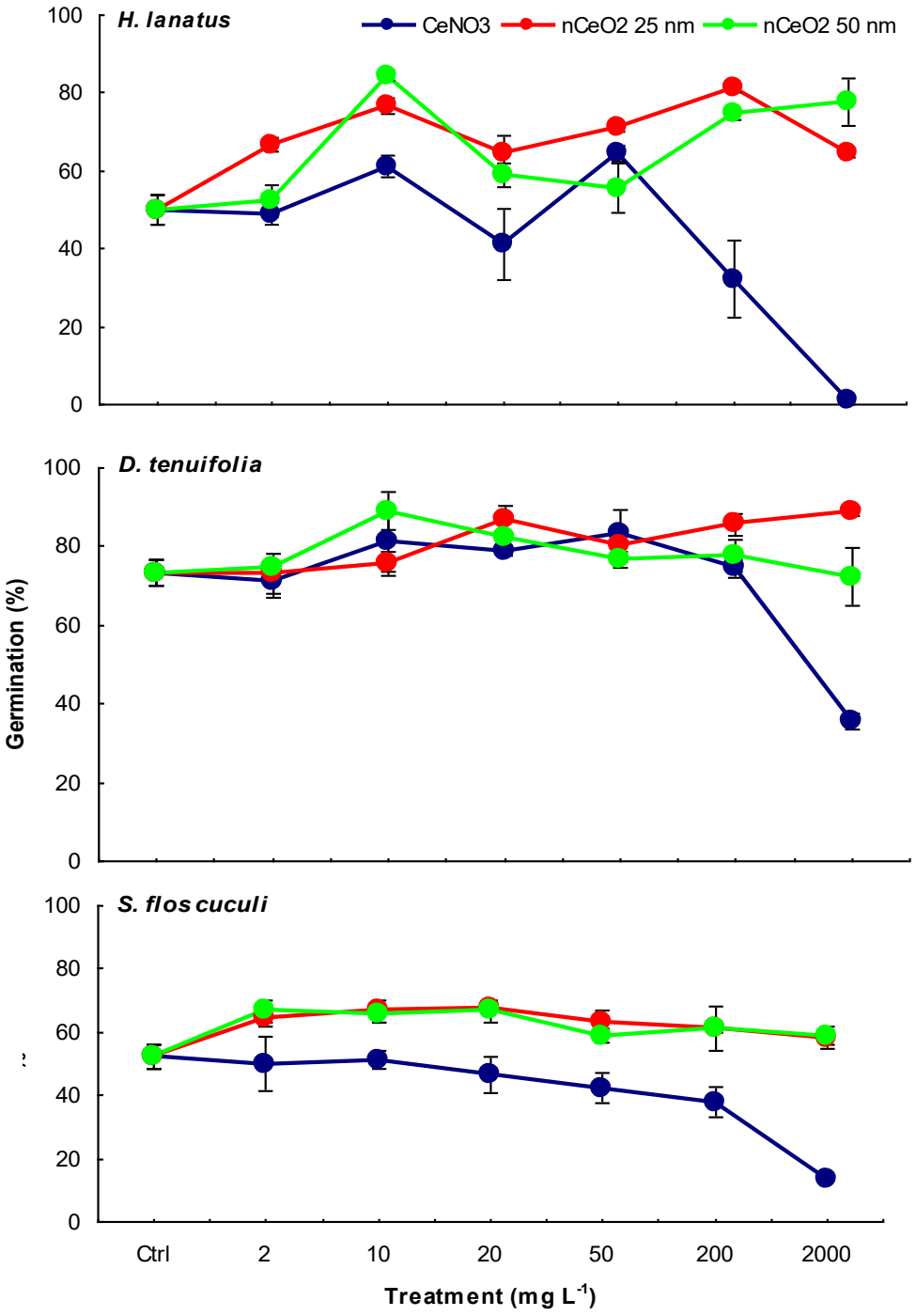


Figure 21: Percentage of seed germination in *H. lanatus*, *D. tenuifolia* and *S. flos cuculi*, grown in Petri dishes and treated with solutions of $CeNO_3$ and $nCeO_2$ (25 nm and 50 nm) at different concentrations (0, 2, 10,20, 50, 200 and 2000 mg L^{-1}).

After the statistical analysis with two – way ANOVA inside the species, considering concentration and class size, the graphics here reported (figure 21) demonstrate that there is not a statistically significant effect in all species for the two class size used of *n*CeO_2 (p = 0.30). Moreover, analysis highlight that, even at high concentrations, seed germination is not affected by Ce nanoparticles: treatments improve the germination percentage in all the 3 species, if compared with control plants (50% in *H. lanatus* and *S. flos cuculi*, 70% in *D. tenuifolia*). In particular in *D. tenuifolia*, germination of control seedlings is 77.33% and the percentage of seeds treated exceeds the 80%, reaching the 86.67%, 85.56% and 88.89%, respectively at 20, 200 and 2000 mg L^{-1} (*n*CeO_2 25 nm); 88.89% and 82.22% at 10 and 20 mg L^{-1} (*n*CeO_2 50 nm). An interesting result derives from $CeNO_3$ data: the trend of germination is similar to *n*CeO_2 at low concentrations (from 2 to 50 mg L^{-1}) but at 200 and 2000 mg L^{-1}, the percentage rapidly decreases, due to the toxic effect of NO_3. In particular, in *S. flos cuculi*, germination of seeds treated with $CeNO_3$ is lower than all the others obtained after *n*CeO_2 treatments and constantly decreases. In *H. lanatus*, at 200 mg L^{-1}, the percentage rapidly decrease and at 2000 mg L^{-1} none of the seeds germinates. The study demonstrates that the same observation described for germination could be extended to another parameter: root elongation. Also for this variable, the reported graphics (figure 22) demonstrate that there is not a statistically significant effect (p = 0.14) in all species for the double class dimensions of *n*CeO_2. In *S. flos cuculi*, the elaborated data are quite completely the same both for 25 nm and 50 nm, in particular at 10 mg L^{-1} (1.70 and 1.65 cm), 50 mg L^{-1} (1.80 and 1.77 cm), 200 mg L^{-1} (1.64 and 1.61 cm) and 2000 mg L^{-1} (1.62 and 1.57 cm). On the contrary, regarding *n*CeO_2 concentrations, data demonstrate that for the development of root tissues,

there is a significant effect (p = 0.05) and it is influenced in the same manner by *n*CeO_2 treatments.

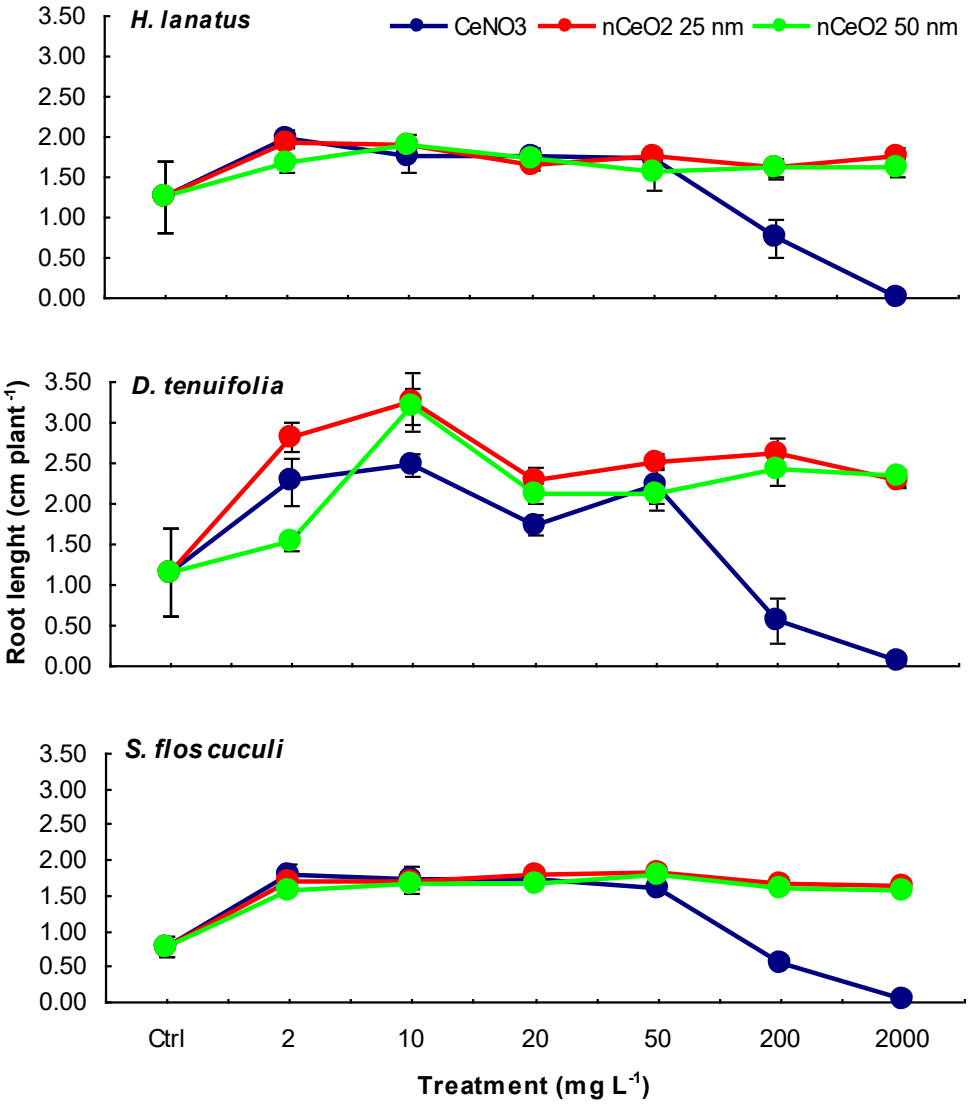


Figure 22: Root elongation in *H. lanatus*, *D. tenuifolia* and *S. flos cuculi*, grown in Petri dishes and treated with solutions of $CeNO_3$ and *n*CeO_2 (25 nm and 50 nm) at different concentrations (0, 2, 10,20, 50, 200 and 2000 mg L^{-1}).

The results demonstrate that, even at high concentrations, root length is not affected by *n*CeO_2: indeed, treatments improve the root elongation in all the three species, if compared with control plants (1.15 cm). In *D. tenuifolia*, the average root length is greater than in the other 2 species, in particular at 10 mg L^{-1} (3.25 cm for 25 nm nanoparticles (NPs) and 3.19 cm for 50 nm NPs). Also for this parameter, an interesting result derives from $CeNO_3$ data: the trend of germination is similar to *n*CeO_2 till 50 mg L^{-1}; for higher concentrations (200 and 2000 mg L^{-1}), the development of root rapidly decreases, due to the toxic effect of NO_3. An important aspect, regarding all the native plants, is that at 2000 mg L^{-1} none of the seeds develops any root (0 cm).

4.3 Total content of cerium in seedling tissues

To quantify the total content of cerium (Ce) that was taken up by seedlings in the 3 plant species, the first step was to analyse with ICP – MS, the samples obtained after the acid digestion. The elaborated data with the total concentration of Ce are presented in table 4 (A), B) and C)). The concentration of total Ce in seedling tissues of *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* shows a dose – response and a different magnitude of accumulation between *n*CeO_2 25 nm and 50 nm. This is confirmed by the fact that 25 nm NPs are taken up more than 50 nm NPs, in particular in *D. tenuifolia* and *S. flos cuculi* and at the two highest concentrations of treatments: 189 ± 26.2 and 114 ± 32.2 mg kg^{-1}(dry weight (DW)) at 200 mg L^{-1}; 1841 ± 137 and 1305 ± 129 mg kg^{-1} DW at 2000 mg L^{-1} in *D. teniufolia*, respectively at 25 nm and 50 nm. In *S. flos cuculi*, the total content of Ce corresponds to 165 ± 13.3 and 128 ± 13.6 mg kg^{-1} DW at 200 mg L^{-1}; 1616 ± 67.8 and 1151 ± 86.6 mg kg^{-1} DW at 2000 mg L^{-1}, respectively at 25 nm and 50nm.

Table 4: Two – way ANOVA measuring the effects of treatments and class size of nCeO$_2$ (25 nm and 50 nm) on cerium (Ce) concentration (mg kg^{-1} (dry weight (DW)) in seedlings of *H. lanatus* A), *D. tenuifolia* B) and *S. flos cuculi* C), grown in Petri dishes and treated with 0, 2, 10, 20, 50, 200 and 2000 mg L^{-1}.

Treatment	$CeNO_3$	nCeO$_2$ 25 nm	nCeO$_2$ 50 nm	
Control (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	
2	1.30 ± 0.45	1.22 ± 0.39	1.57 ± 0.51	
10	6.46 ± 1.55	6.72 ± 1.98	9.56 ± 0.56	
20	19.5 ± 1.43	11.3 ± 1.42	16.4 ± 1.86	
50	45.2 ± 3.78	28.4 ± 9.16	25.7 ± 9.28	
200	197 ± 12.0 *	70.4 ± 3.96 *	61.9 ± 9.59 *	
2000	2046 ± 69.4 **	588 ± 45.3 **	416 ± 97.6 **	A)

Treatment	$CeNO_3$	nCeO$_2$ 25 nm	nCeO$_2$ 50 nm	
Control (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	
2	1.84 ± 0.37	0.30 ± 0.25	0.17 ± 0.05	
10	8.61 ± 0.72	2.83 ± 0.91	4.94 ± 0.80	
20	17.4 ± 1.42	10.5 ± 2.52	12.2 ± 1.04	
50	43.6 ± 3.65	32.7 ± 12.8	18.6 ± 7.43	
200	191 ± 17.3 *	189 ± 26.2 *	114 ± 32.2 *	
2000	2081 ± 42.3 **	1841 ± 137 **	1305 ± 129 **	B)

Treatment	$CeNO_3$	nCeO$_2$ 25 nm	nCeO$_2$ 50 nm	
Control (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	
2	1.66 ± 0.14	0.91 ± 0.15	0.78 ± 0.30	
10	8.14 ± 0.60	6.38 ± 1.80	6.23 ± 1.35	
20	17.0 ± 1.02	9.40 ± 1.81	11.4 ± 1.90	
50	43.1 ± 2.41	32.5 ± 6.55	30.5 ± 5.18	
200	186 ± 3.08 *	165 ± 13.3 *	128 ± 13.6 *	
2000	2154 ± 112 **	1616 ± 67.8 **	1151 ± 86.6 **	C)

A statistically significant effect of treatments ($p = 0.004$) in Ce accumulation in seedlings is verified for all the three species at 200 and 2000 mg L^{-1}. This observation is demonstrated by Ce concentration that linearly increases with

treatments and this is extremely clear in plants treated with $CeNO_3$. On the contrary, at higher concentrations (200 and 2000 mg L^{-1}) of *n*CeO_2 25 nm and 50 nm, the total content of Ce is lower than at the same treatment of $CeNO_3$. For example, in *H. lanatus*, the concentrations measured at 2000 mg L^{-1} are 2047 ± 64.9 mg kg^{-1}DW, 589 ± 45.3 mg kg^{-1} DW and 417 ± 97.6 mg kg^{-1} DW (respectively for $CeNO_3$, *n*CeO_2 25 nm and 50 nm). This aspect could be justified by the fact that, in particular at high concentrations, nanoparticles tend to form consistent aggregates, because of the major interactions and attraction, due to van der Waals forces or chemical bonds. For this reason, it is more difficult for roots to uptake these aggregates.

4.4 Effects of *n*CeO_2 on biometric variables

After the experiment described above, regarding the influence of *n*CeO_2 on seed germination and root elongation of the three species, a new experiment was set up to evaluate the impact and the effects of CeO_2 nanoparticles on these spontaneous plant species during their life cycle and to highlight the uptake and possible translocation of cerium (Ce) in the above – ground plant biomass. The common aim between the previous experiment and the latter, is to evaluate if there is a possible effect connected to different concentrations and double class size of *n*CeO_2, in this case on biometric variables. This new experiment was carried out in a greenhouse, trying to reproduce with a pot soil experiment, a condition similar as much as possible to reality. Five days after sowing, seeds of the three plant species germinated both in control and in soil treated with *n*CeO_2, without any particular visible differences in treated plants. In this first part of the results about the pot soil experiment, the effects of *n*CeO_2 on *H. lanatus* and *D. tenuifolia* will be described.

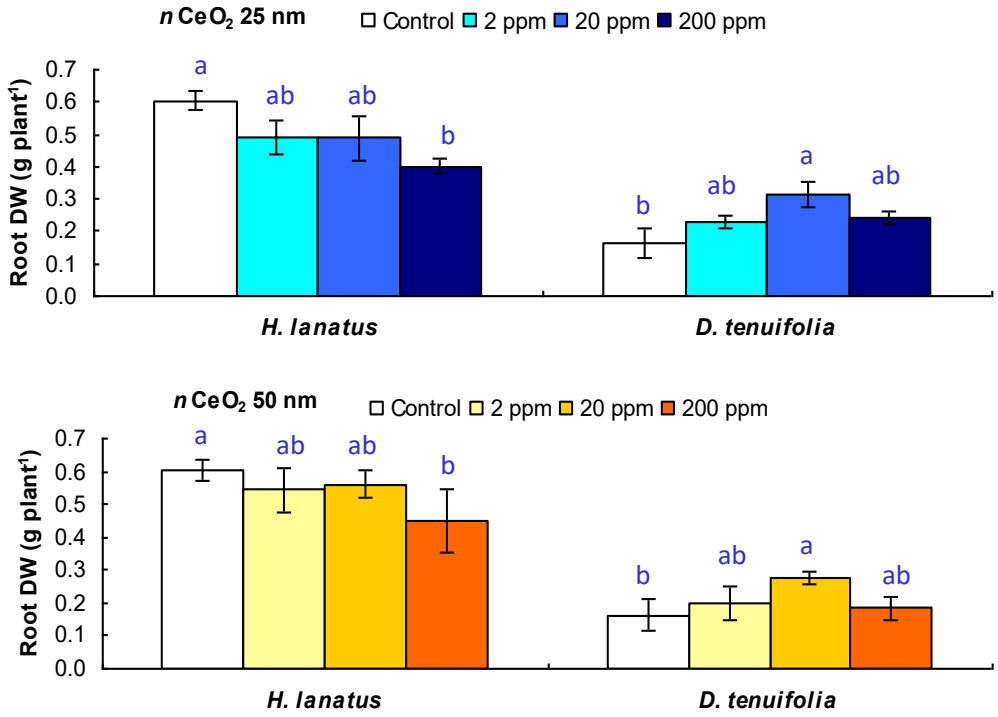


Figure 23: Root dry weight of *H. lanatus* and *D. tenuifolia* treated with different concentrations (0, 2, 20 and 200 mg L^{-1}) and different class size (25 nm and 50 nm) at the end of 60 days – greenhouse experiment.

After the statistical analysis of data with two - way ANOVA within species (considering concentrations and class size), it has been decided to represent and describe the most important biometric variables. As expected, the behaviour of *H. lanatus* and *D. tenuifolia* to the treatments is completely different, because of the species belonging to different botanical families (*H. lanatus* is a monocotyledonous and *D. tenuifolia* is a dicotyledonous). Considering the graphics reported in figure 23 about roots (dry weight (DW)), ANOVA indicates that the double class size of NPs does not significally influence the development of roots in both species ($p = 0.28$ in *H. lanatus*; $p = 0.24$ in *D. tenuifolia*). On the other hand, ANOVA also shows significant differences inside

the species regarding treatments (p = 0.04 in *H. lanatus*; p = 0.02 in *D. tenuifolia*). It is observed that treatments have a negative effect in *H. lanatus*, because in control plants, root apparatus is more developed than in treated ones. Indeed, the highest value corresponds to control and it is 0.60 ± 0.03 g DW. On the opposite, *n*CeO_2 solutions increase the root growth in *D. tenuifolia*, with the maximum (highest) development at 20 mg kg^{-1} (0.31 ± 0.04 g DW (25 nm)). Finally, because of different species, a general consideration could be drown about root apparatus: it is more developed in *H. lanatus* than in *D. tenuifolia*. Another biometric variable that was considered in this study is the number of stems. Considering the graphics below (figure 24) also for this parameter, ANOVA demonstrates that the double class dimensions of CeO_2 NPs do not significantly influence the number of stems in both species (p = 0.45 in *H. lanatus*; p = 0.47 in *D. tenuifolia*). In this case, the treatments applied stimulate the sprouting in *H. lanatus* if compared to plants irrigated only with water: ANOVA shows significant differences inside this species regarding treatments (p = 0.03). The higher values are obtained at 20 mg kg^{-1} (20.17 ± 1.59 N. $plant^{-1}$ and 19.08 ± 2.60 N. $plant^{-1}$, respectively at 25 nm and 50 nm). Conversely, treatment solutions do not have significant effects in *D. tenuifolia* (p = 0.06). For this reason, when comparing the number of stems in control plants with that of treated ones (higher value), it turns out that they are very similar (3.40 ± 0.5 g N. $plant^{-1}$ in control; 4.8 ± 0.3 N. $plant^{-1}$ in 20 mg kg^{-1} (50 nm)). These extremely different values between *H. lanatus* and *D. tenuifolia* could be due to intrinsic characteristics of the species, where *H. lanatus* tend to sprout and produces several secondary shoots stimulated by *n*CeO_2, while *D. tenuifolia* develops one main steam and only a few secondary stems.

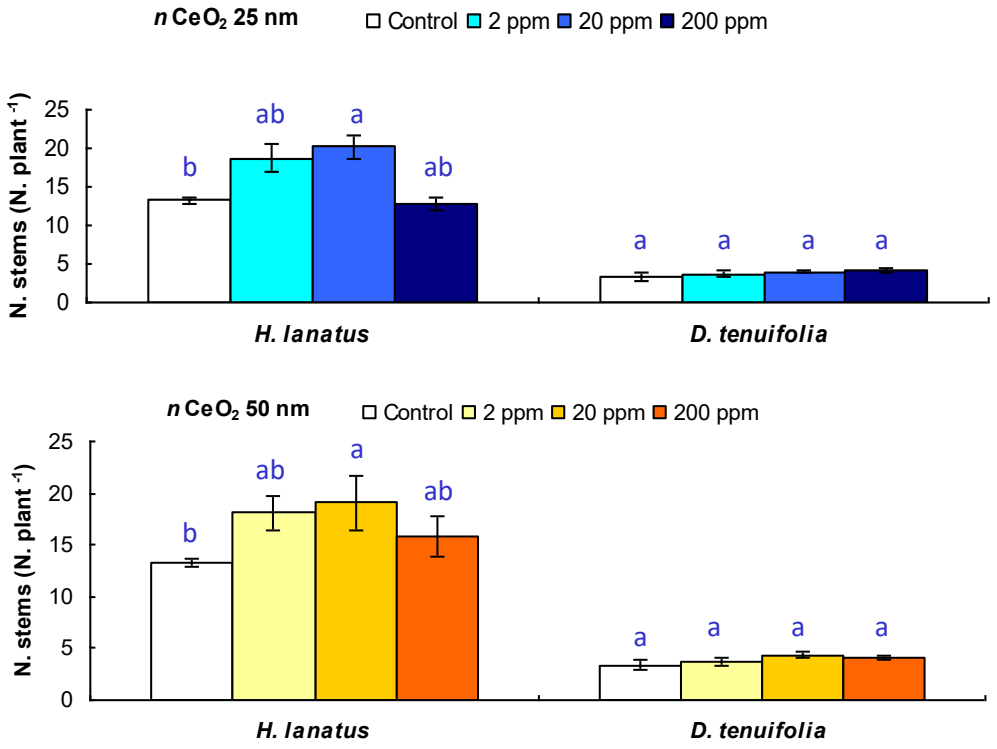


Figure 24: Number of stems in *H. lanatus* and *D. tenuifolia* treated with different concentrations (0, 2, 20 and 200 mg L^{-1}) and different class size (25 nm and 50 nm) at the end of 60 days – greenhouse experiment.

Leaf area is an important biometric variable, considered very often in studies regarding plant growth. Comparing the behaviour of the two species, a statistically significant difference could be evidenced about treatments ($p = 0.02$ in *H. lanatus*; $p = 0.0002$ in *D. tenuifolia*). It is observed that treatments stimulate growth and leaf development in both species (figure 25), if compared with plants irrigated only with water (366.4 ± 35.6 cm^2 $plant^{-1}$ and 220.9 ± 14.7 cm^2 $plant^{-1}$ in control, respectively in *H. lanatus* and *D. tenuifolia*). The highest values are observed in both species in plants, that were treated with a solution of 20 mg L^{-1}, but in *H. lanatus* at 25 nm (602.3 ± 83.8 cm^2 $plant^{-1}$) and in *D.*

tenuifolia at 50 nm (312.7 ± 11.2 cm^2 $plant^{-1}$). In the two species, for the leaf area, the lower values correspond to plant grown without treatments.

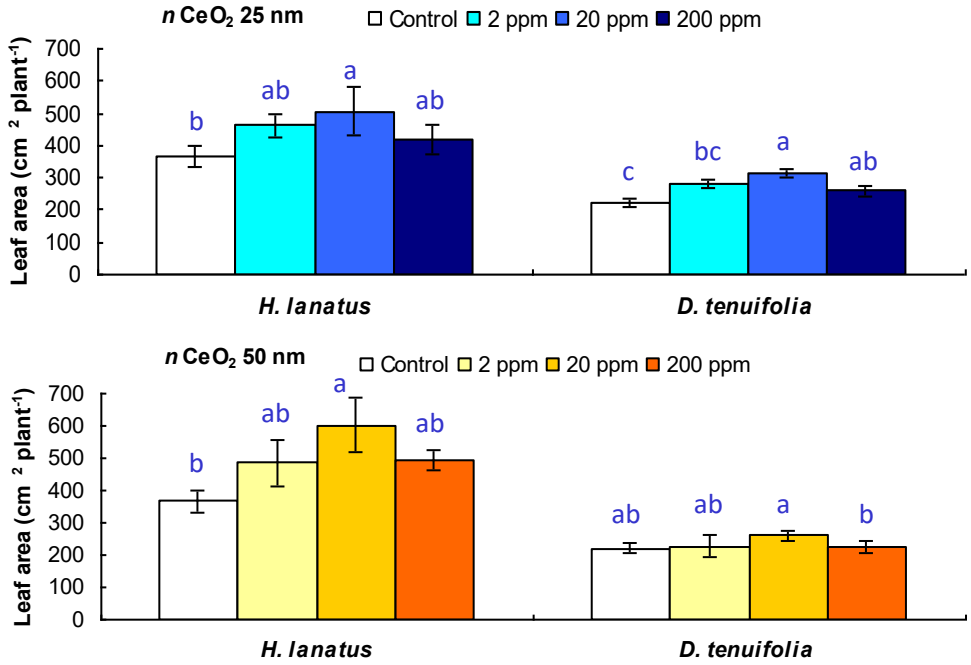


Figure 25: Leaf area in *H. lanatus* and *D. tenuifolia* treated with different concentrations (0, 2, 20 and 200 mg L^{-1}) and different class size (25 nm and 50 nm) at the end of 60 days – greenhouse experiment.

The analysis of variance (ANOVA) indicates also for this parameter that the double class size of NPs does not influence the development and growth of leaves in both species (p = 0.08 in *H. lanatus*; p = 0.81 in *D. tenuifolia*).

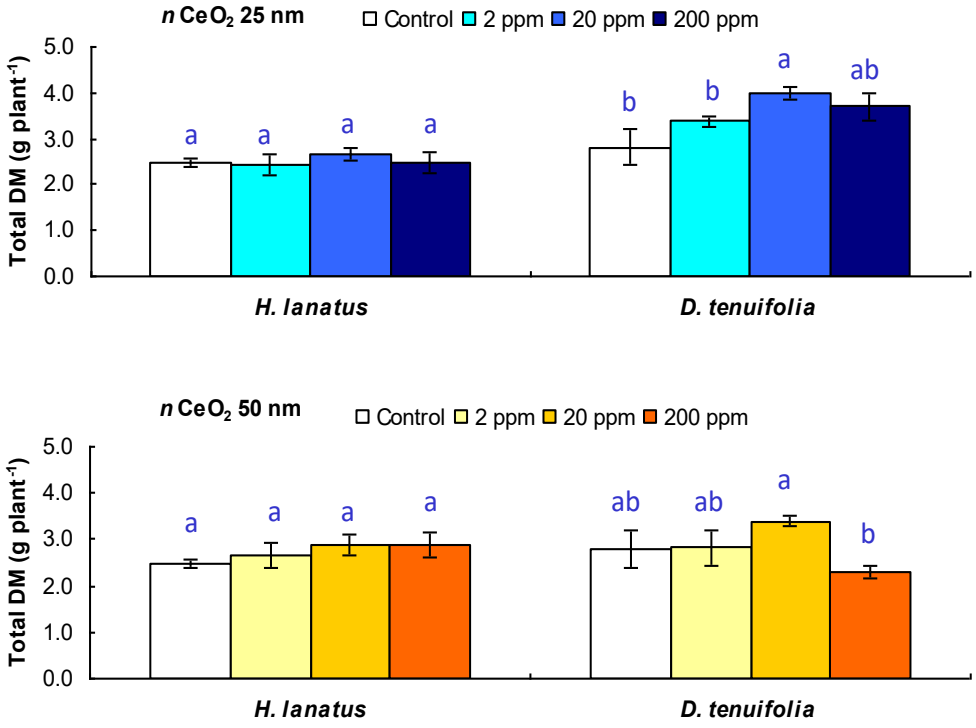


Figure 26: Total dry matter in *H. lanatus* and *D. tenuifolia* treated with different concentrations (0, 2, 20 and 200 mg L^{-1}) and different class size (25 nm and 50 nm) at the end of 60 days – greenhouse experiment.

Regarding the total dry matter, this is an important biometric variable used to demonstrate a possible alteration of plants growth, connected to treatments. Also in this case, the behaviour of the two species is completely different: in *H. lanatus*, ANOVA highlights that both treatments and $nCeO_2$ class sizes of NPs do not have significant influence on this parameter ($p = 0.26$). Indeed, in control, the total dry matter is 2.48 ± 0.11 g $plant^{-1}$ and the highest value in this species is 2.89 ± 0.23 g $plant^{-1}$, corresponding to the treatment with 20 mg kg^{-1} (50 nm). On the contrary, in *D. tenuifolia*, treatments stimulate the development and the growth of plants and so they have a positive effect ($p =$

0.006) in particular at 20 mg kg^{-1}. The statistical analysis demonstrates that also for this specie, there is no effect on total dry matter due to $nCeO_2$ class size (p = 0.36).

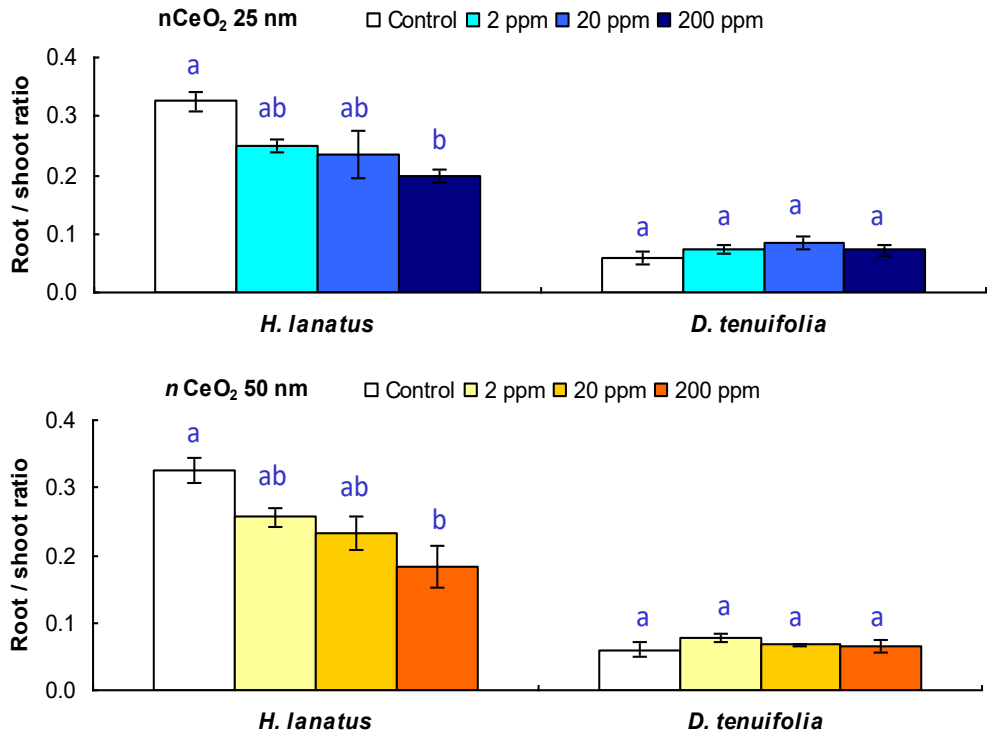


Figure 27: Root / shoot ratio in *H. lanatus* and *D. tenuifolia* treated with different concentrations (0, 2, 20 and 200 mg L^{-1}) and different class size (25 nm and 50 nm) at the end of 60 days – greenhouse experiment.

The last variable considered in this experiment is the root / shoot ratio (referred to dry weight): as it could be expected comparing the results in the parameters previously described, *H. lanatus* and *D. tenuifolia* have a completely different behaviour, probably justified by the fact that they belong to two different botanical families. Considering the graphics reported in figure

27, ANOVA indicates that the double class size of NPs does not significantly influence the ratio in both species (p = 0.44 in *H. lanatus*; p = 0.33 in D. *tenuifolia*). On the other hand, ANOVA also shows significant differences within *H. lanatus* regarding treatments (p = 0.02), but not in *D. tenuifolia* (p = 0.17). It could be observed that treatments have a clear negative effect in *H. lanatus*, because in plants wetted only with water, the ratio is higher than in treated ones. Indeed, the highest value of this parameter corresponds to the control and it is 0.33 ± 0.02. On the opposite, *n*CeO_2 solutions do not influence the ratio in *D. tenuifolia*, because in control it corresponds to 0.06 ± 0.01 and the highest value is reached in plants treated with solution of *n*CeO_2 at 20 mg kg^{-1} (0.09 ± 0.01 (25 nm)).

4.4.1 Effects of repeated applications of *n*CeO_2 on *Silene flos cuculi* L.

The other sub - experiment, carried out under the same conditions in the greenhouse and trying to reproduce with a pot soil experiment a condition similar as much as possible to reality, included repeated treatments of *n*CeO_2 in *S. flos cuculi*, at concentrations of 0, 20 and 200 mg kg^{-1} respectively after 20 and 40 days. The *n*CeO_2 class size chosen for this experiment was 25 nm, because easily absorbed by plants. With this part of the pot soil experiment, it was tried to evaluate if there are effects on this spontaneous plant specie during its life cycle, connected to a situation of chronic "contamination" and prolonged exposure to nano ceria. The biometric parameters considered are roots dry weight and leaf area, because the objective of this simultaneous study is to highlight the possible toxicity caused by *n*CeO_2. Regarding the root dry weight, it decreases with additional treatments (figure 28), both 20 mg kg^{-1}

(0.38 ± 0.05 g plant^{-1} DW (T0) and 0.27 ± 0.05 g plant^{-1} DW after the last treatment (T40)) and 200 mg kg^{-1} (0.32 ± 0.02 g plant^{-1} DW after T0 and 0.23 ± 0.04 g plant^{-1} DW after T40). Nevertheless, the dry weight of treated plants is similar of that observed in control (0.29 ± 0.02 g plant^{-1}). On the opposite, the results about the leaf area (figure 28), even if present much more variability than in those of root DW, demonstrate that this biometric variable is not influenced by treatments after 20 and 40 days from sowing (535 ± 147 cm^2 plant^{-1} (T0) and 528 ± 99.6 cm^2 plant^{-1} (T40) at 20 mg plant^{-1}; 459 ± 63.6 cm^2 plant^{-1} (T0) and 569 ± 104 cm^2 plant^{-1} (T40) at 200 mg plant^{-1}). This is an interesting result because it highlights that there is no translocation of *n*CeO_2 from the root – soil system to the above – ground sections of *S. flos cuculi* (table 5).

Table 5: Total content of Ce (mg kg^{-1}) observed at the end of 60 days – greenhouse experiment in roots and leaves of *S. flos cuculi*, grown in control soil and *n*CeO_2 25 nm amended soil (after 20 days (T20) and 40 days (T40)) with different concentrations (0, 20 and 200 mg L^{-1}).

Roots

Treatment	T 0	T 20	T 40
Control (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20	2.01 ± 0.55	2.69 ± 0.83	3.89 ± 1.57
200	6.26 ± 1.23	9.42 ± 3.01	11.9 ± 3.90

Leaves

Treatment	T 0	T 20	T 40
Control (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20	0.04 ± 0.01	0.21 ± 0.03	0.49 ± 0.18
200	0.38 ± 0.23	0.58 ± 0.15	1.06 ± 0.90

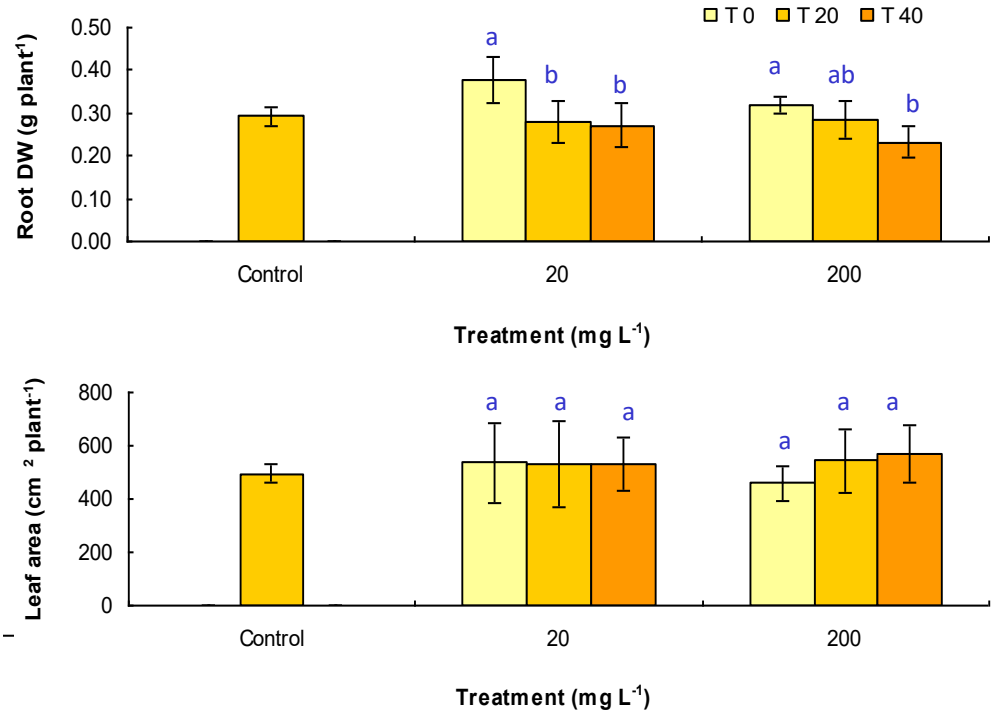


Figure 28: Root dry weight and leaf area in *S. flos cuculi* after additional treatment (T0, 20 days (T20) and 40 days (T40)) with different concentrations (0, 20 and 200 mg L^{-1}) of *n*CeO_2 25 nm at the end of 60 days – greenhouse experiment.

4.5 Total content of cerium in plant fractions

Quantifying the total content of Ce among the different tissues in both plant species is the fundamental passage to do after acid digestion of samples. Roots and leaves of control and CeO_2 NPs treated samples of *H. lanatus* and *D. tenuifolia* were analysed with ICP – MS and the elaborated data with the total concentration of cerium are presented in table 6 and 7.

Table 6: Ce concentration (mg kg^{-1}) observed at the end of 60 days – greenhouse experiment in roots and leaves of *H. lanatus* and *D. tenuifolia*, grown in control soil and *n*CeO_2 amended soil with different concentrations (2, 20 and 200 mg L^{-1}), corresponding to the total content of CeO_2 25 nm in roots and leaves.

Roots			Leaves		
Treatment	***H. lanatus***	***D. tenuifolia***	**Treatment**	***H. lanatus***	***D. tenuifolia***
Control	0.07 ± 0.03 b	0.01 ± 0.00 c	**Control**	0.01 ± 0.00 b	0.01 ± 0.00 c
2 ppm	0.60 ± 0.25 b	0.30 ± 0.17 b	**2 ppm**	0.07 ± 0.06 ab	0.24 ± 0.19 a
20 ppm	1.31 ± 0.07 b	0.31 ± 0.08 bc	**20 ppm**	0.85 ± 0.30 a	0.05 ± 0.01 b
200 ppm	16.08 ± 3.23 a	9.39 ± 1.62 a	**200 ppm**	0.16 ± 0.11 b	0.10 ± 0.07 bc

Table 7: Ce concentration (mg kg^{-1}) observed at the end of 60 days – greenhouse experiment in roots and leaves of *H. lanatus* and *D. tenuifolia*, grown in control soil and *n*CeO_2 amended soil with different concentrations (2, 20 and 200 mg L^{-1}), corresponding to the total content of CeO_2 50 nm in roots and leaves.

Roots			Leaves		
Treatment	***H. lanatus***	***D. tenuifolia***	**Treatment**	***H. lanatus***	***D. tenuifolia***
Control	0.07 ± 0.03 b	0.01 ± 0.00 c	**Control**	0.01 ± 0.00 b	0.01 ± 0.00 c
2 ppm	0.23 ± 0.07 b	0.17 ± 0.04 bc	**2 ppm**	0.07 ± 0.02 ab	0.06 ± 0.03 a
20 ppm	1.15 ± 0.28 b	0.34 ± 0.09 b	**20 ppm**	0.83 ± 0.17 a	0.04 ± 0.01 b
200 ppm	5.27 ± 1.11 a	1.12 ± 0.17 a	**200 ppm**	0.50 ± 0.13 b	0.26 ± 0.11 bc

A statistically significant effect of treatments is observed for roots both in *H. lanatus* ($p = 0.0001$) and in *D. tenuifolia* ($p = 0.0002$). The concentration of total Ce in tissues of *D. tenuifolia* shows a dose – response effect and a different magnitude of accumulation between *n*CeO_2 25 nm and 50 nm. This confirms the fact that, not only in the previous study of germination, that was set up in a controlled environment (Petri dishes) but also in the pot soil study, 25 nm NPs

are taken up more than 50 nm NPs. The obtained data could be due to the direct contact of root with the amended soil, where plants grew for 60 days. In the germination experiment, *D. tenuifolia* absorbed higher quantities of Ce in seedlings, while in the 60 – days greenhouse study, *H. lanatus* presents a greater quantity of metal, both in roots and in leaves fractions and for all treatments. In both species, it is observed that the highest quantity of Ce is measured in roots, treated with solutions of 200 mg L^{-1}: 16.08 and 9.39 mg kg^{-1} (25 nm) and 5.27 and 1.12 mg kg^{-1} (50 nm), respectively in *H. lanatus* and *D. teniufolia*. This observation demonstrates that Ce concentration linearly increases with treatments and this is extremely clear in plants treated with 25 nm solutions. Leaves are the plant fraction where cerium content is the lowest and presents more variability in data, maybe because leaves are the most distal part of plants. From the obtained results, it could be affirmed that in these spontaneous species, there is almost no translocation of cerium from root – soil system to the other plant fraction and this could be an important ecological aspect, in particular for potential contaminated situations.

5. DISCUSSION

The rapid increase in the use of *n*CeO_2 in many application areas has caused the spread of these nanoparticles (NPs) in different environmental matrices. In recent years, researches have tried to understand the relationships between NPs and plants: for this reason, the aim of this research is to illustrate how NPs could affect plants and the possible effects that NPs could have on the plant life cycle. A preliminary experiment to verify the uptake and of *n*CeO_2 by seedlings and about the possible internalization of *n*CeO_2 in plants was particularly necessary for this study, because it is one of the first that has tried to analyse the relationship between NPs and native / spontaneous plant species. Indeed, until now, the majority of studies concern crops and cultivated species. The results obtained in this experiment from the enzymatic digestion of seedlings highlight that all the three species (*H. lanatus*, *D. tenuifolia* and *S. flos cuculi*) absorb CeO_2 NPs both 25 nm and 50 nm. The higher number of particles observed in *S. flos cuculi* could be explained by the smaller dimensions of seeds, if compared with the other two species. Therefore, seeds of *S. flos cuculi* have a greater surface area in direct contact with treatment solutions, used for seed germination in Petri dishes. For this reason, *n*CeO_2 could be absorbed by roots and then transported to the above – ground parts of seedlings. This observation can also be found in other studies regarding Au NPs (Dan et al., 2015), Ag NPs (Bao et al., 2016), CeO_2 NPs (Dan et al., 2016) and TiO_2 NPs (Kurepa et al., 2010). The results obtained are valuable in terms of comparisons between the three plant species studied and there are no particular differences regarding the particle size distribution between 25 nm and 50 nm. This is a particular aspect because suggests that 25 nm NPs could be subject to an agglomeration process inside plants after absorption. After

entering through roots, NPs quite probably move to other parts of the seedlings through the vascular system and form aggregates, as reported by Bao et al., (2016) and Corredor et al., (2009). Generally, the agglomeration has been attributed to the attraction between nanoparticles, caused by van der Waals forces or chemical bonds. It is currently not clear how nanoparticles create agglomerates in plants: it may depend on the characteristics and properties of nanoparticles and on the biological aspects of the considered plant species. For example, the presence of aggregates has been described for Ag NPs in *Arabidopsis* plants (Bao et al., 2016), but there are also some studies that demonstrate that NPs do not have this type of alteration, as for Au NPs in tomato plants (Dan et al., 2015). In addition, scientific literature highlights that in some plants the agglomeration happens before the passage from roots to the other parts of seedlings and a few other studies have verified that NPs do not undergo any other process during the transportation. After SP – ICP – MS analysis, the results point out that the highest frequency (number of peaks) is observed in *S. flos cuculi*, followed by *D. tenuifolia* and *H. lanatus*. Since the seedlings of all three of the plant species grew in Petri dishes with the same treatment concentration, it could be affirmed that the uptake and translocation process of *n*CeO_2 in these spontaneous species are different, maybe due to intrinsic features of the different botanical families and further research is needed in the future.

In scientific literature, there are several studies and publications that assess the positive and negative effects of ENMs on terrestrial plants (Monica and Cremonini, 2009), but the majority of them are set in model conditions, such as Petri dishes and hydroponics, and do not try to reproduce a real environmental situation (Gardea – Torresdey et al., 2014; Miralles et al., 2012; Ma et al., 2010; Rico et al., 2011). There is still a lack of knowledge of the relationship between

NMs and plants, stemming from plant species, plant fraction, particle size and type, concentration and exposure (Rico et al., 2011; Miralles et al., 2012; Burman et al., 2013a; Burman et al., 2013b; Kumar et al., 2015). The experiments described in this thesis highlight the effects of *n*CeO_2 on native plants, from early stages to adult plants. Cerium is among the ten most used ENMs in the world (Piccinno et al., 2012) and is the subject of different experiments regarding the interactions with plants. *n*CeO_2 generally enters plants through root uptake and may cause several effects on the early stages of plant development, such as reducing or increasing germination rates, and improving, reducing or inhibiting radical growth (Lopez - Moreno et al., 2010a). The previous studies in literature carried out in controlled conditions, reported that the toxicity of NMs in the initial development stages of plant growth could be due to physico – chemical properties, as well as particle size and shape (Yang and Watts, 2005; Lin and Xang, 2007). The results of the first experiment of this dissertation highlight the effects on germination and root length of seeds of spontaneous plants species, treated at different concentrations and with different sizes of cerium oxide nanoparticles. The 25 nm and 50 nm class size did not reduce the seed germination in *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* if compared with control. Moreover, in agreement with Rico et al., (2015b), the different treatments used in this study do not affect the percentage of germination in the three species, neither at low concentrations nor at 2000 mg L^{-1}, when in *D. tenuifolia*, germination reaches the 90% (50 nm), which is more than double the amount of this parameter in $CeNO_3$ at the same concentration. Interestingly, an aspect concerning *n*CeO_2 solutions is that they stimulate germination more than $CeNO_3$ starting from low concentrations. This is in contrast with the results obtained by Lopez – Moreno et al., (2010a) at 2000 mg L^{-1}, who noted that cerium nanoparticles inhibited the germination in

corn, tomato, maize and cucumber, but do not have particular effects on alfalfa. In addition, an interesting aspect to be highlighted from this work of thesis concerns $CeNO_3$ results: the trend of germination is similar to the trend of seeds watered with *n*CeO_2 solutions at low concentrations, but at 200 and 2000 mg L^{-1}, the percentage of germination rapidly decrease, due to the toxic effect of NO_3. In *H. lanatus*, at 2000 mg L^{-1} none of the seeds germinates. A possible explanation could be due to the low tolerance of *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* to *n*CeO_2. In a very similar study, Lopez - Moreno et al., (2010b) also studied the influence of cubic *n*CeO_2 on seed germination of soybean seedlings and no significant effects on germination were reported, except at the highest treatment (2000 mg L^{-1}). Other researchers that studied the effects of *n*CeO_2 on germination on different crops are Andersen et al., (2016), that focus on onion, oat, cabbage, cucumber, carrot soybean, lettuce, tomato and corn: no acute toxicity effect was observed in the first step of plant development. Moreover, germination of barley was not affected by *n*CeO_2, even at 2000 mg L^{-1} (Mattiello et al., 2015), confirming the results highlighted by Lopez – Moreno et al., (2010b) and in agreement with Rico et al., (2015b). In general, ENMs show early negative consequences on development stages of crops (Miralles et al., 2012; Gardea – Torresday et al., 2014) and this observation is confirmed in some publications for ZnO nanoparticles (Lin and Xin, 2008; Priester et al., 2012; Prasad et al., 2012; Yoon et al.,2014). Seed germination is negatively affected by ZnO (Stampolius et al., 2009), but the same type of nanoparticles inhibits the previously described parameter in corn (Lin and Xin, 2007). On the contrary, no significant effect was observed on seed emergence of wheat and radish exposed to ZnO (Lopez – Moreno et al., 2010). TiO_2 is another type of nanoparticles, used in several experiments and it improves the percentage of germination in spinach (Zheng et al., 2005), wheat

(Hassen et al., 2012), rice, cucumber, radish, tomato and pea (Boonyanitipong et al., 2011; Wu et al., 2012; Song et al., 2013a; Fan et al., 2014). These are important considerations as they assert the complexity of studying the "world" of NMs, their different effects and their potential risks from the agricultural and ecological point of view, not only at a high trophic level, but from the first stages of plant development. The other parameter measured in this type of study is root elongation: scientists found that in several plants, root development is inhibited by NPs, as Yang and Watts (2005) demonstrate in corn, cucumber and carrot, using Al NPs. In most of the experiments concerning early stages of plant development, researchers use different types of NPs but with the same class size. In the experiments of this thesis, therefore, two classes of dimensions have been used from the beginning of the studies. Curiously, the same observation described for germination of *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* could be extended to root elongation. Indeed, there is no particular effect on root length in all the species studied, due to the solutions applied during the experiment, containing double class dimensions of *n*CeO_2 NPs. On the contrary, regarding *n*CeO_2 concentrations, the development of root tissues is influenced and in particular is stimulated even at high concentrations (2000 mg L^{-1}). Also, for this parameter, an interesting result derives from the comparison between $CeNO_3$ and *n*CeO_2: root length is not affected by *n*CeO_2, while the development of the root rapidly decreases, due to the toxic effect of $CeNO_3$. An important aspect regarding all the native plants here analysed is that at 2000 mg L^{-1} of $CeNO_3$, none of the seeds develops any roots. In literature, there are reports with contradictory evidence: Lopez - Moreno et al. (2010a) report that the root growth in maize and cucumber seedlings is significantly promoted by *n*CeO_2, also at high concentrations, while the same treatments have negative effects on root development in alfalfa and

tomato. In a similar study, Ma et al., (2010) verify that in lettuce, there is a reduction of root length but the growth of root in cabbage, cucumber, radish, rape and tomato are not affected by 2000 mg L^{-1}. On the other hand, an inhibitory effect of ZnO in zucchini and soybean is reported respectively by Yoon et al., (2014) and Stampoulis et al., (2009). A negative effect of *n*TiO_2 on root elongation in cucumber is also highlighted Mushtaq (2011), while Boonyanitipong et al., (2011) do not record any effect on root length in rice seedlings exposed to *n*TiO_2.

Some papers also give information about the total content of Ce inside seedling tissues, after the exposure to *n*CeO_2. In this way, it may be possible to individuate the root uptake of the NPs used, depending on the biological characteristics of the plant species and intrinsic properties of NPs. In this thesis, the study concerning the concentration of total Ce in seedling tissues of *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* shows a dose – response and a different magnitude of accumulation between *n*CeO_2 25 nm and 50 nm. This is confirmed by the fact that 25 nm NPs are taken up more than 50 nm NPs, in particular in *D. tenuifolia* and *S. flos cuculi*. This is in agreement with Zhang et al., (2011), which verifies that cucumber roots absorbed higher amounts of 7 nm NPs than 25 nm NPs of *n*CeO_2. It is also observed that Ce concentrations linearly increase with the increasing concentration of the treatments and this is evident, especially in plants treated with $CeNO_3$. On the contrary, at higher concentrations (200 and 2000 mg L^{-1}) of *n*CeO_2 25 nm and 50 nm, the total content of Ce is lower than at the same treatment of $CeNO_3$. This aspect could be justified by the fact that, in particular at high concentrations, nanoparticles tend to agglomerate due to van der Waals forces or chemical bonds and it is more difficult for roots to take up these aggregates.

Different scientific studies and reviews have tried to describe, analyse and give information about the effects of NMs in higher terrestrial plants, but most of them focus the attention only on the early stages and do not cover the full life cycle. Indeed, Gardea – Torresday et al., (2014) demonstrate that there are only thirty studies that face the effects of NMs on the entire life cycle of plants and only five concern *n*CeO_2. The plants studied are tomato (Wang et al., 2012), cilantro (Morales et al., 2013), rice (Rico et al., 2013a), wheat (Rico et al., 2014) and radish (Corral – Daiaz et al., 2014). One of the aims of this thesis is to study Ce uptake and translocation, and the effects on biometric variables in spontaneous species, under *n*CeO_2 exposure, because they have rarely been studied before and are almost completely unknown. In recent years, different studies focus the attention on the effects that *n*CeO_2 have on plant growth and physiology in agriculture (Collin et al., 2014). For this reason, the potential interactions of *n*CeO_2 and crops are investigated not only in early stages of development but also over the entire life cycle. Contradictory effects are clearly obtained, also in this case depending on the type of NM, the plant species, the growth media and the exposure, because not all parameters are affected in the same manner (Hawthrone et al.,2014; Majumdar et al.,2014; Collin et al., 2010). The experiment described in the thesis is carried out in a greenhouse where soil was amended with *n*CeO_2 solutions before sowing, in attempt to reproduce a condition as similar possible to the open field with a pot soil experiment. The results obtained in the experiment previously described in this dissertation and regarding the effects of *n*CeO_2 on biometric variables highlight that the double class size of NPs does not influence the development of roots and the same evidence could be extended to the number of stems, the leaf area, the root / shoot ratio and the total dry matter of *H. lanatus* and *D. tenuifolia*. On the other hand, the different concentrations have

contradictory effects on the same variables, probably due to the fact that the species belong to different botanical families. In particular, in *H. lanatus*, treatments have a negative effect on the development of roots because root dry weight is lower in treated plants than in the control ones, but in *D. tenuifolia*, *n*CeO_2 solutions increase the root growth, with the maximum value obtained at 20 mg kg^{-1}. A particular consideration could be made about root apparatus, because in spite of the treatments, it is more developed in *H. lanatus* than in *D. tenuifolia*. Hawthorne et al., (2014) also demonstrate significant reduction in zucchini root fresh mass on exposure to 1000 mg kg^{-1} of CeO_2 in soil. In *S. flos cuculi*, the initial treatment improves development of roots, if compared with control plants, but repeated treatments cause the decrease of root dry weight, in particular at 200 mg kg^{-1}, maybe due to a toxic effect of *n*CeO_2. A similar study regarding lettuce shows an enhanced dried root mass at 100 mg kg^{-1} but a reduction at 1000 mg kg^{-1} (Gui et al., 2015). Another biometric variable which is not always considered in other studies is the number of stems: in this case, the treatments applied stimulate the sprouting in *H. lanatus* compared to plants irrigated only with water. On the contrary, treatment solutions do not influence this variable in *D. tenuifolia*, in which the number of stems of control plants is very similar to treated ones. These extremely different values between *H. lanatus* and *D. tenuifolia* could be due to intrinsic characteristics of species, where *H. lanatus* tends to sprout and produces several secondary shoots stimulated by *n*CeO_2, while *D. tenuifolia* develops the main stem and only a few secondary stems. Also in repeated treatment experiment of *S. flos cuculi*, this variable is not particularly influenced, even in plants that grow in soil amended with 200 mg kg^{-1} *n*CeO_2. In agreement with this results about minimal effects of 1000 mg kg^{-1} *n*CeO_2 on stems, Zhao et al., (2013) and Morales et al., (2013) highlight that no particular

effects are observed around plant growth, respectively in cucumber and cilantro. Conversely, *n*CeO_2 enhance the development of stems and biomass at 500 mg kg^{-1} (Lopez – Moreno et al., 2010). Leaf area is an important biometric variable, very often considered in studies regarding plant grow. Treated plants of *H. lanatus* and *D. tenuifolia* are stimulated in growth and leaf development compared with plants irrigated only with water, interestingly showing lower values corresponding to plant grown without treatments. Also, in *S. flos cuculi*, leaf area is not influenced by repeated treatments, neither at low dosage nor after a 60 – days experiment, during which, plants were subjected to two additional treatments at 200 mg kg^{-1}. Hawthrone et al., (2014) describe how at low concentrations of *n*CeO_2 up to 1000 mg kg^{-1}, zucchini shows a significant reduction of leaf area, but they have a reversal trend upon exposure to 1000 mg $kg^{-1.}$ An important biometric variable that is not specified or described in different studies regarding the plant life cycle is the root / shoot ratio. In the experiment of this thesis, as could be expected, the effects of *n*CeO_2 on *H. lanatus* and *D. tenuifolia* are opposite and the species behave in a completely different way. As indicated, it is observed that treatments have a clear negative effect in *H. lanatus*, because in control plants, the ratio is higher than in treated ones. Indeed, the highest ratio is measured in plants grown in untreated soil. An opposite behaviour is found in the repeated dose experiment, where *S. flos cuculi* plants show an increase root / shoot ratio after the additional treatments, both at 20 and 200 mg kg^{-1}. The interesting result is that the values obtained in treated plants at the end of the greenhouse study are quite similar to those of controls. Conversely, *n*CeO_2 solutions do not influence the ratio in *D. tenuifolia*, because the results of control and treated plants have quite the same values. Some of the previously described statements of this experiment, find correspondence in other papers of scientific literature: in studies by Vittori

Antisari et al., (2015), tomato plants are used as a model and *n*CeO_2 treatments do not affect their growth; however a decrease in dry biomass is observed. In addition, Ma et al., (2016) study the physiological modifications at different growth stages in turnip and *n*CeO_2 do not enhance plant biomass. Finally, Zhang et al., (2017) and Servin et al., (2017) study the effects of *n*CeO_2 at different concentrations in lettuce and they highlight that at higher concentrations (2000 mg kg^{-1}), *n*CeO_2 caused a decrease of fresh and dry weight of plant fractions. Thus, the studies previously described suggest that *n*CeO_2 in the soil media could cause alterations in some biometric parameters in different plant species, but do not caused evident toxic effects on plants, even if exposed for prolonged period of time. This could be due to the chemical stability *n*CeO_2 in the soil. For these reasons, the similar results obtained both in the experiment with *H. lantaus* and *D. tenuifolia* and in the study with *S .flos cuculi* predicting additional treatments after 20 and 40 days, could be explained by the fact that NMs tend to have a longer residence time in soil than in aquatic media (Peijnenburg et al., 2010). Moreover, NMs interact with soil components and the behaviour of particles with soil physicochemical properties is still partially unknown (Collin et al., 2014; Park et al., 2016). It is important to underline that matrix plays an important role in NMs transport from the soil to the roots and the upper part of plants (Esfandyari et al., 2015; Fujita and Kobayashi, 2016). The results of both the sub – experiments where plants of *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* grew in organic soil show relatively high concentrations of Ce in roots only in correspondence of the highest treatment (200 mg kg^{-1}), while leaves have very low content for all the treatments. This trend is observed in all the plant species considered in this thesis. Other studies also reported higher accumulations of NPs in roots and limited transport to shoots (Wang et al., 2013; Hong et al., 2015; Lui and

Kottke, 2004; Bao et al., 2016). In addition, other studies demonstrate that *n*CeO_2 tend to stay in soil or in the root – soil system (Wang et al., 2012; Zhao et al., 2012a; Schwabe et al., 2013; Chichiricco and Poma, 2015). These pot soil experiments with spontaneous species confirm that the fate and the uptake of NPs in plants depend on the type of plant and the growth environment. Zhao et al., (2012a) demonstrate that Ce translocation in corn plants depends on the soil organic matter, since plants grown in soil with low organic matter are found to present more Ce in tissues than plants grown in organic matter rich soil. Barrios et al., (2015) affirm that in their study, only at high values of nCeO_2 in soil, Ce concentration in stems and leaves is higher than the control, so no treatment shows particular Ce concentration in above ground tissues. Trujillo - Reyes et al., (2013) also expose radish to nCeO_2 and demonstrate that there is no translocation in plants. In general, plants which are not hyper – accumulators store excessive metals in the roots to protect themselves against metal toxicity (Liu and Kottke, 2004).

6. CONCLUSIONS

Several studies in literature demonstrate that engineered nanomaterials (ENMs) could influence the entire life cycle of plants. The effects are sometimes opposite and depend on the plant species and on the type of ENMs. The majority of the studies in literature concern agricultural and cultivated species, with particular attention to stimulation – inhibition of germination, root elongation, plant development and consequently seed production. From these assumptions, in this dissertation has investigated spontaneous plant species, in an attempt to describe how *n*CeO_2 with two class sizes could affect these plants and the possible effects that ENMs could have on their life cycle. This is maybe the first study focusing attention on species that are native and widespread in different ecological conditions. The experiment results evidence that *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* are able to uptake and translocate *n*CeO_2 NPs from roots to the above – ground parts of seedlings, with different mechanisms. In addition, the study suggests that nanoparticles (NPs) undergo a process of agglomeration in all the three species. Moreover, *n*CeO_2 treatments improve the percentage of germination in *H. lanatus*, *D. tenuifolia* and *S. flos cuculi* if compared with plants grown only with MilliQ. In addition, root elongation is not affected at low concentrations, nor at 2000 mg L^{-1}. Hence, there is no evidence of toxicity in the early stages of plant development. The different class size does not induce statistically significant effects on the three species in the first stage, nor during the entire life cycle. An important result for seedlings concerns $CeNO_3$, which almost totally inhibits the early stages of plants at higher concentrations. During the entire life cycle, the three species behave in different ways after treatments, because the parameters analysed are affected both in negative and positive ways. Indeed,

H. lanatus reduces its root development, but on the contrary, *D. tenuifoli*a improves the growth of the same parameter. On the other hand, *S. flos cuculi*, after being watered with additional solutions of *n*CeO_2, tends to inhibit plant root development, but not in a significant way, so it could not be justified as an effect of cerium toxicity. Both in *H. lanatus* and in *D. tenuifolia*, treatments improve the vegetative growth of the upper part and this aspect could be verified in particular with leaves. The experiment with repeated applications of *n*CeO_2 does not demonstrate any particular type of translocation of Ce from the root – soil system to leaves, perhaps due to the stability of Ce in soil. This aspect could justify why in literature, the fate and the effects of ENMs in the soil – plant system are still poorly understood. The obtained results suggest that the three plant species could be used to recover cerium from the environment, which is very important because cerium is one of the most produced and widely utilized ENMs in Europe. Future studies should focus on how ENMs affect plant metabolism and growth in a complex system, as similar as possible to real conditions, because the majority of the experiments are currently conducted in hydroponic systems or in Petri dishes. In addition, there should be interest in further knowledge of the effects of ENMs on the soil microbial community and further studies of the possible transfer of these new materials not only in plants but also along the food chain. This experiment could contribute to risk assessment of NPs present not only in edible plants but also in spontaneous species. In addition, this study could be useful for more in – depth knowledge about the interactions between ENMs and plants but more in general with the environment; the behaviour, the fate and in particular the effects of these materials on spontaneous plant species and natural matrices need to be studied more in detail. Therefore, similar studies could reveal important information for future ecological risk assessment efforts, in

particular for countries where there are no laws that regulate the presence of these new materials in the environment. Indeed, they are becoming the new contaminants in the future.

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