

Article

Influence of Cerium Oxide Nanoparticles on Two Terrestrial Wild Plant Species

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Abstract: Most current studies on the relationships between plants and engineered nanomaterials (ENMs) are focused on food crops, while the effects on spontaneous plants have been neglected so far. However, from an ecological perspective, the ENMs impacts on the wild plants could have dire consequences on food webs and ecosystem services. Therefore, they should not be considered less critical. A pot trial was carried out in greenhouse conditions to evaluate the growth of *Holcus lanatus* L. (monocot) and *Diplotaxis tenuifolia* L. DC. (dicot) exposed to cerium oxide nanoparticles ($n\text{CeO}_2$). Plants were grown for their entire cycle in a substrate amended with $200 \text{ mg kg}^{-1} n\text{CeO}_2$ having the size of 25 nm and 50 nm, respectively. $n\text{CeO}_2$ were taken up by plant roots and then translocated towards leaf tissues of both species. However, the mean size of $n\text{CeO}_2$ found in the roots of the species was different. In *D. tenuifolia*, there was evidence of more significant particle aggregation compared to *H. lanatus*. Further, biomass variables (dry weight of plant fractions and leaf area) showed that plant species responded differently to the treatments. In the experimental conditions, there were recorded stimulating effects on plant growth. However, nutritional imbalances for macro and micronutrients were observed, as well.

Keywords: cerium oxide nanoparticles; *Holcus lanatus*; *Diplotaxis tenuifolia*; biometric variables; Ce accumulation



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1. Introduction

Nanotechnology has revolutionized the manufacturing aspects in several fields of applications. The tunable chemical and physical properties of engineered nanomaterials (ENMs) are still improving electronics, energy, aerospace, human health, innovative materials, and many others [1]. Despite the current and expected great benefits of nanotechnologies, an unintended side effect is the uncontrolled release of ENMs. Due to involuntarily releases, tons of ENMs are discharged into the environment [2]. According to quantitative models, soils and freshwaters are the endpoints of such materials [3–5]. Such evidence raises concern on the impact of ENMs on the ecosystems and biota since we still have patchy knowledge on this issue [6].

Cerium oxide nanoparticles ($n\text{CeO}_2$) are included in the top 10 most produced and widely utilized nanomaterials globally; their global estimated production is 10,000 metric tons per year [7]. The global emission of $n\text{CeO}_2$ in soil was estimated to be 129–1029 metric tons per year [8]. The impact of $n\text{CeO}_2$ is already under observation in different environmental matrices and ecosystems [9,10]. It has been reported a $n\text{CeO}_2$ concentration in soil in the range $0.09\text{--}1.12 \text{ mg kg}^{-1}$ [11].

So far, data are available in the scientific literature regarding the adverse effects on plant metabolism and growth due to ENMs exposure [12–14]. On the other hand, convincing demonstrations of the positive effects of ENMs applied to plants as fertilizers were already provided [15]. However, due to the vastness of the investigation field, the knowledge acquired is still instead fragmentary for some reasons. Early studies were carried out without specific reference protocols and different conditions (e.g., hydroponics, synthetic soil, potting soil, or agricultural soil), obtaining different results. For obvious reasons (e.g., food and fodder production, agriculture management, and social and financial issues), most studies have been conducted on food crops [16].

Concerning $n\text{CeO}_2$ —plants relationships, investigations have been carried out in short-term experiments and controlled conditions (i.e., Petri dishes and hydroponic systems) on germinating seeds or very young plant specimens [17]. In these experimental conditions, studies have been carried out on *Triticum aestivum* [18], *Zea mays* [19], *Hordeum vulgare* [20,21], *Glycine max* [22], *Cucumber sativus* [23], *Raphanus sativus* [24], and *Lycopersicon esculentum* [25–29].

From a wider perspective a tiny number of plant species was investigated so far. In fact, crops represent about 0.1% of the vascular plants known to science [30]. Spontaneous plant species are not less important. Being soil one of the endpoints of ENMs, it is of the utmost importance to consider the interactions with plants, to be intended not only as food producers but as primary producers and ecosystem services providers [31].

Beyond crops, ENMs-plant relationships were investigated on some spontaneous species. More aquatic and wetland species have been studied than terrestrial ones [32–34]. Regarding terrestrial ecosystems, as far as we know, *Pinus sylvestris* L. and *Quercus robur* L. are the only no-food terrestrial plant species investigated for the exposure to respectively nano-Ag and $n\text{CeO}_2$ [35].

The aim of this work is to evaluate the influence of $n\text{CeO}_2$ with different particle size on the entire growth cycle of *Holcus lanatus* (L.) and *Diplotaxis tenuifolia* (L.) (DC) (dicot). The grass *H. lanatus* (monocot) is a hairy, tufted, fibrous-rooted and meadow soft perennial grass, growing between 50 and 100 cm tall, belonging to Poaceae family [36]. It occurs over a wide range of soil types and fertility conditions. It 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. The dicot *D. tenuifolia* is a Brassica perennial flowering plant, native in Europe and West Asia. It is commonly found in different habitats but in particular in ruderal plant associations, in abandoned fields and ruderal areas, more rarely in cultivated fields [37]. The species have been chosen since they are widespread in natural systems, highly competitive, and easily adaptable to different ecological conditions.

2. Results

2.1. Nanomaterial Characterization

The hydrodynamic diameter (Hd) distribution of both $n\text{CeO}_2$ 25 nm and 50 nm is in agreement with the value provided by the supplier. Both materials exhibit a monodisperse size particle distribution in the nanometric range with relatively low polydispersity index (PDI) (0.17 and 0.25) and the main size peak at 62.0 nm and 91.0 nm, respectively. The relative Z-averages sizes were found to be much larger than these values (127 nm and 206 nm, respectively, $n\text{CeO}_2$ 25 nm and 50 nm), indicating particle aggregation. Since a high net surface charge is typically associated with weak nanoparticles interactions and aggregation, these data are coherent with the higher Z-average size detected for sample $n\text{CeO}_2$ 50 nm with respect to $n\text{CeO}_2$ 25 nm (ζ -potentials 39.2 mV (25 nm) and 24.1 mV (50 nm)). Particle field observations of $n\text{CeO}_2$ suspensions are reported in Figure 1.

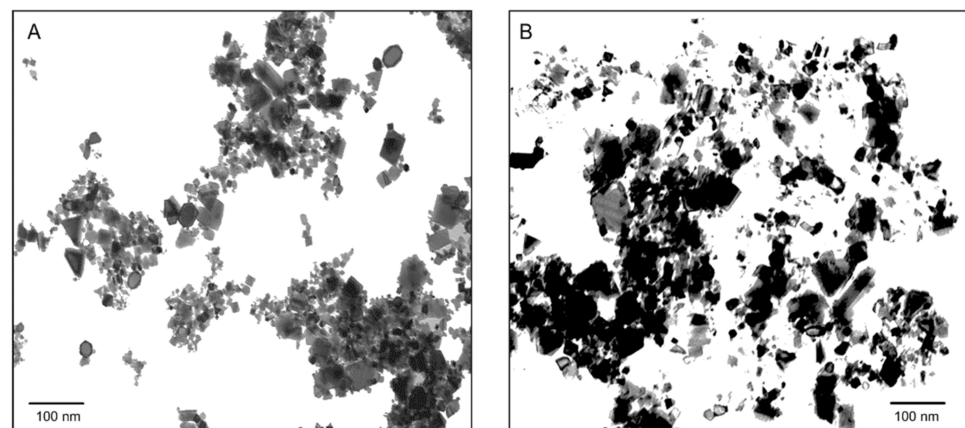


Figure 1. TEM image of $n\text{CeO}_2$ 25 nm (A) and 50 nm (B) suspensions.

2.2. $n\text{CeO}_2$ in Plant Tissues

Before illustrating and discussing the experimental results, it is necessary to prove whether the $n\text{CeO}_2$ has been taken up by plant roots and possibly translocated towards the aerial parts. Plants grew in the presence of different concentrations of $n\text{CeO}_2$ in the soil medium. The early step of this study was to verify the entry of $n\text{CeO}_2$ into plant tissues and verify if any particle size transformations into the plant tissues occurred. Roots and leaves of *H. lanatus* and *D. tenuifolia* were subjected to the enzyme extraction procedure, and sp-ICP-MS analyzed the extracts. The data obtained from the sp-ICP-MS analysis, reported in Figure 2 and Table S1, provide two early results.

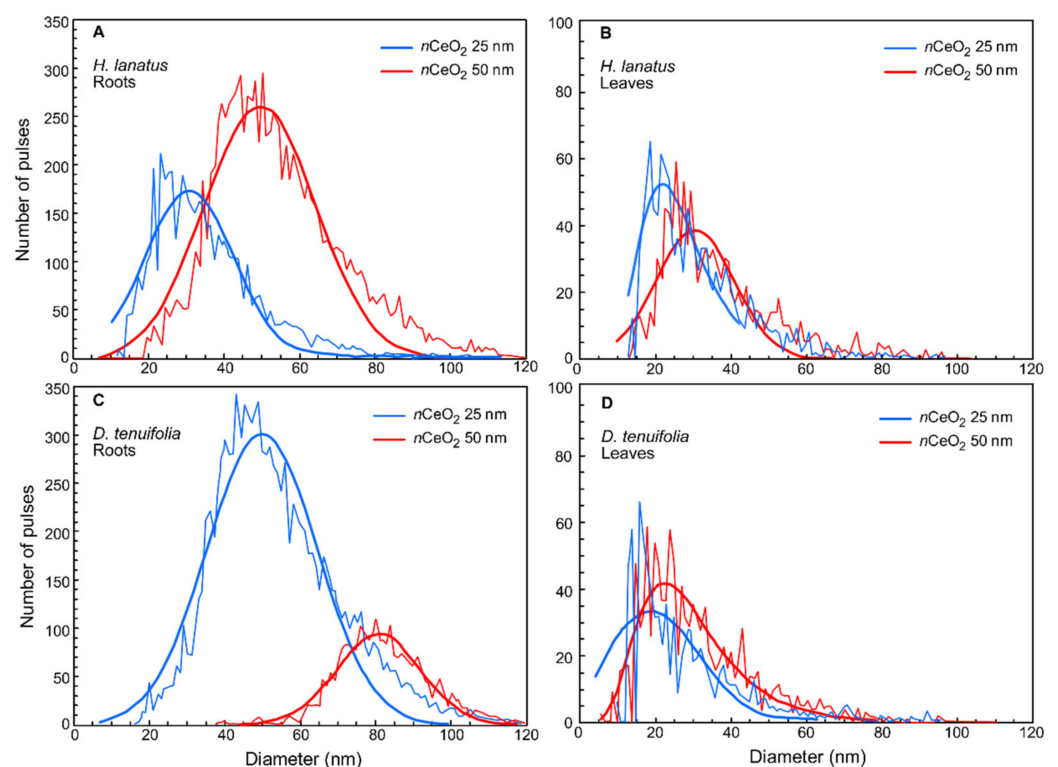


Figure 2. Particle size distribution of $n\text{CeO}_2$ 25 nm (blue) and 50 nm (red) extracted by enzymatic digestion procedure from roots (A) and leaves (B) of *H. lanatus*, and from roots (C) and leaves (D) of *D. tenuifolia*.

The first is that $n\text{CeO}_2$ were found in the root tissues and leaves of both species. The second is that $n\text{CeO}_2$ root uptake occur differently in the two species, with consequences

on $n\text{CeO}_2$ size and the dissolution of Ce ions inside the leaf tissues. In particular, in the roots of *H. lanatus* the most frequent $n\text{CeO}_2$ size (30 nm and 51 nm) and the mean particle size (36 nm and 56 nm) were very close to the actual size of Ce nanoparticles (25 nm and 50 nm, respectively) (Figure 2A and Table S1). After entering the root tissues, $n\text{CeO}_2$ has not undergone aggregation and could move to the aerial plant fractions through the vascular tissues. A small fraction of dissolved Ce ion ($7.07 \mu\text{g L}^{-1}$) was detected in the root extracts containing the larger $n\text{CeO}_2$, whereas it was practically absent in plants treated with $n\text{CeO}_2$ 25 nm (Table S1). In roots of *D. tenuifolia* the right-shift of fitting curves (Figure 2C) compared to previous ones indicate particle aggregation. In fact, the most frequent particle size was 50 nm and 79 nm while the mean particle size was 53 nm and 82 nm that are values higher than the reference size, 25 nm and 50 nm (Figure 2C and Table S1). By observing the curves reported in Figure 2A–C, we can appreciate that in the roots of *D. tenuifolia* was detected a much greater number of small $n\text{CeO}_2$ and a much smaller number of larger particles compared to *H. lanatus*.

$n\text{CeO}_2$ were also extracted from leaf tissues of both species (Figure 2B–D). However, the sp-ICP-MS analyses showed that fewer particles were accumulated in the leaves than the ones found in the roots, but they were also smaller in size (range 26–36 nm) (Figure 2B–D and Table S1). Finally, a negligible concentration of dissolved Ce was found in the leaf extracts for both species. So, such smaller $n\text{CeO}_2$ did not undergo dissolution after having reached the aerial plant fractions (Table S1).

2.3. Plant Growth

The following biometric variables were detected on the fractions of *H. lanatus* and *D. tenuifolia*: root dry matter, number of stems per plant, stems dry matter, leaves dry matter, leaf area per plant, and total plant dry matter. A two-way ANOVA was carried out to evaluate the effects of the experimental factors on the mentioned plant growth variables (Table S2).

An overall view of the results shows clearly that the response of the species to $n\text{CeO}_2$ was different. For the biometric variables, except leaves dry mass, a statistically significant effect of the factor “species” was observed. A statistically significant interaction “species X treatment” on root dry matter ($p = 0.0368$ *) and the number of stems per plant ($p = 0.0157$ *) were observed, as well.

The aerial biomass production of plants responded to the presence of $n\text{CeO}_2$. The effect of the “treatment” factor was statistically significant in the case of the number of stems per plant ($p = 0.0094$ **), for the leaf area ($p = 0.0005$ **) and the dry weight of the leaves ($p = 0.0482$ *). The effect of the treatment on the dry weight of the stems was not statistically significant. However, a p -value of 0.0574—very close to the significance threshold—indicates that this variable is consistent with the previous evidence (Table S2).

The root dry mass, number of stems per plant, leaf area, and leaves dry mass per plant are presented in Figure 3A–D. The results of the post-hoc comparisons Tukey HSD test for each species are reported, as well.

There was no significant change in the root dry mass of *H. lanatus* ($p = 0.1154$) and *D. tenuifolia* ($p = 0.3096$) in response to the treatments (Figure 3A). This is likely due to the buffer effect of the potting soil, which—at the not very high concentration of 200 mg kg^{-1} $n\text{CeO}_2$ —hid any effect on the root growth. Figure 3B reports the number of stems per plant. Tillering, i.e., the production of secondary stems, is a very important phenological phase of monocotyledon species. In addition to producing a large number of secondary stems compared to *D. tenuifolia*, *H. lanatus* was also clearly stimulated by $n\text{CeO}_2$ 50 nm, which supported the increase of stem number per plant by 31% ($p = 0.0269$ *).

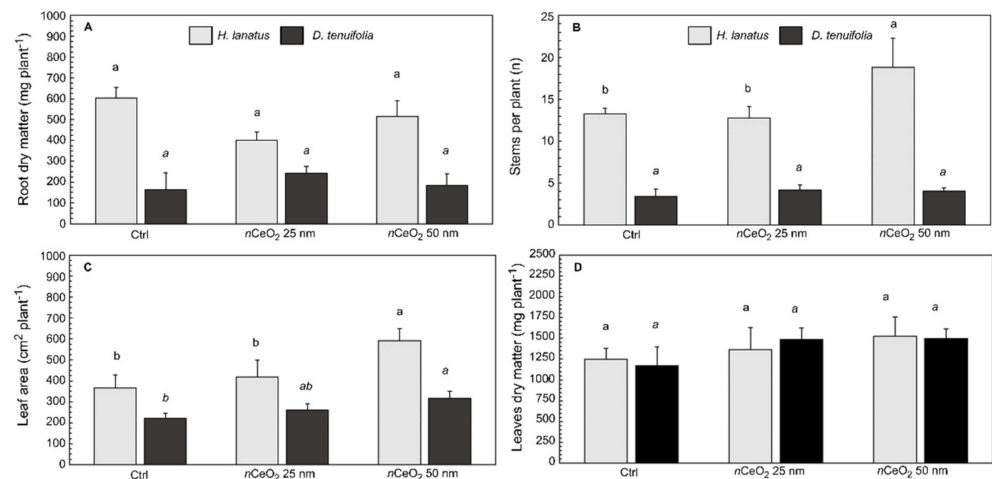


Figure 3. (A) Root dry matter, (B) number of stems per plant, (C) leaf area and (D) leaf dry matter \pm standard deviation of *H. lanatus* and *D. tenuifolia*. Comparison between control and plants grown in presence of 200 mg kg^{-1} nCeO₂ having respectively 25 nm and 50 nm. For each species the statistically significant difference ($p \leq 0.05$) between treatments is indicated by the letters using one-way ANOVA follow by Tukey's test.

Since the treated plants had a greater vegetative development than the control ones, it is reasonable to expect an increase in leaf apparatus. This has happened in terms of leaf area (Figure 3C). In *H. lanatus*, the treatment effect with nCeO₂ 25 nm was not intense enough to be statistically detectable by the ANOVA. In contrast, nCeO₂ 50 nm resulted in an increase of 66% and 41% for the control and nCeO₂ 25 nm treated plants, respectively. In *D. tenuifolia* the response to nCeO₂ treatments in terms of leaf area is even more evident. Plants raised in the substrate amended with 25 nm nCeO₂ develop a greater leaf area than the control (+18%, 261 vs. 221 cm² plant⁻¹), while treatment with nCeO₂ 50 nm promoted a 44% increase (317 cm² plant⁻¹) compared to control (Figure 3C). Finally, according to the increase of leaf area, the leaves dry mass responded to the treatment. The ANOVA indicated a statistically significant effect of the factor "treatment" ($p = 0.0482$ *) and the post-hoc test showed that nCeO₂ 25 nm and 50 nm stimulated a higher biomass production in leaves than control plants (1210 mg kg⁻¹), with an increase of respectively 17% (1420 mg kg⁻¹) and 24% (1511 mg kg⁻¹). However, analyzing separately the results of the two species, it can be verified that data variability hides the effect of the size of nCeO₂, which is not statistically significant for both *H. lanatus* and *D. tenuifolia* compared to control (Figure 3D).

The biomass allocation in plants was affected by nCeO₂ treatments, as well. Data analysis for these variables followed the same approach used for the biometric variables (Table S3). As happened for the measured variables, the effect of the factor "species" was always statistically significant at 2-way ANOVA, whereas the "treatment" was statistically significant for R/S ratio ($p = 0.0038$ **), RMF ($p = 0.0070$ **), and LAR ($p = 0.0021$ **) (Table S4). In this case, we deepened the data analysis with particular attention to the effect of nCeO₂ size. As expected, taking into account the biometric data, the ratios that describe the biomass allocation in the fractions of *D. tenuifolia* did not respond to the treatments. In fact, for each parameter, the treatment with 25 nm and 50 nm nCeO₂ did not produce statistically significant effects compared to the control plants (Figure 4A–D). Oppositely, in *H. lanatus* we observed statistically significant differences between treated and untreated plants. In addition, 2-way ANOVA showed a significant interaction "species" \times "treatment" respectively in R/S ratio and RMF. According to the measured variables, statistics carried out for each species indicated that *H. lanatus* always responded to the nCeO₂ treatments.

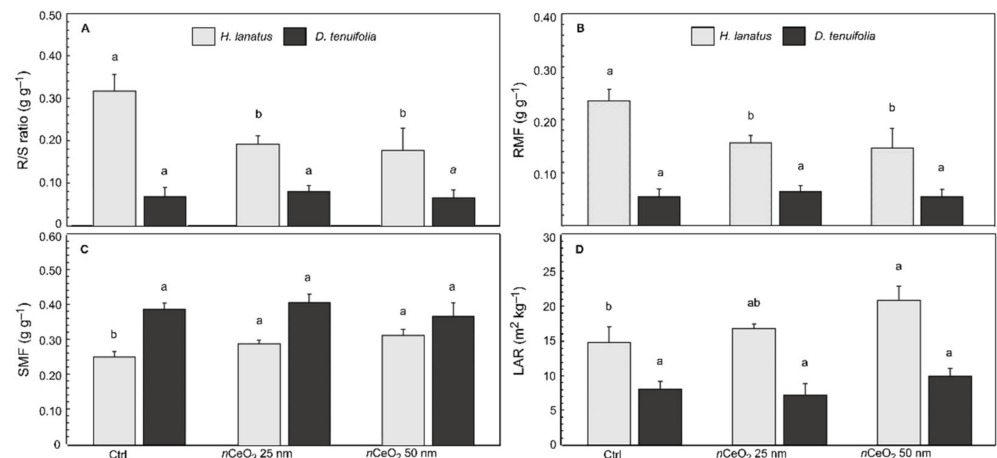


Figure 4. (A) Root to shoot ratio (R/S ratio), (B) Root mass fraction (RMF), (C) Stem mass fraction (SMF) and (D) Leaf area ratio (LAR) \pm standard deviation of *H. lanatus* and *D. tenuifolia*. Comparison between control and plants grown in presence of 200 mg kg^{-1} $n\text{CeO}_2$ having respectively 25 nm and 50 nm. For each species the statistically significant difference ($p \leq 0.05$) between treatments is indicated by the letters according the Tukey's test. —1

R/S ratio—and its inverse—are frequently used for an early assessment on the distribution of plant biomass. Data indicate that $n\text{CeO}_2$ treated *H. lanatus* suffered a reduction of about 40% of the R/S ratio ($p = 0.0085$ **) compared to the control plants (Figure 4A), confirming the previously observed $n\text{CeO}_2$ stimulating action on aerial biomass. The corresponding reduction in the allocation of biomass in the root system of *H. lanatus* is indicated in Figure 4B, where RMF shows a statistically significant reduction with respect control (-35.7% , $p = 0.0105$ *). In both cases, no statistically significant differences were observed in response to the different sizes of $n\text{CeO}_2$ (Figure 4A–B).

In the case of SMF, the statistical analysis did not detect significant effects of the treatment on the accumulation of biomass in the drums ($p = 0.1022$ ns). This data contradicts the data on the increase in the number of stems in *H. lanatus* plants observed previously (Figure 4B). This is a consequence of the significant difference in the vegetative habitus of monocots compared to dicots. However, in the average of the species, the ANOVA did not reveal the expected effects on the SMF. Once again, after isolating the two species, the stimulating effect of $n\text{CeO}_2$ in *H. lanatus* was highlighted ($p = 0.0054$ **) as it can be appreciated in Figure 4C. Therefore, being the increase of the stem biomass in treated plants statistically significant compared to the control ones (+ 17% averaging the Ce particle size), we can confirm the previous data.

The LAR also detected the effects of $n\text{CeO}_2$ on plant canopy. In broad terms, this rate's meaning can be interpreted as the plant's investment of biomass in photosynthetic tissues to enhance light interception and carbon fixation. Since “the more a plant invests in leaf area, the higher the total carbon gain and the faster growth will be”, we found an increase in LAR in response to the presence of $n\text{CeO}_2$ ($p = 0.0178$ *). More interesting is the fact that *H. lanatus* responded differently to the treatments. Compared to the control ($14.8 \text{ m}^2 \text{ kg}^{-1}$), we observed an increase of 12.8% ($16.7 \text{ m}^2 \text{ kg}^{-1}$) and 40% ($20.8 \text{ m}^2 \text{ kg}^{-1}$), respectively, for $n\text{CeO}_2$ 25 nm and 50 nm (Figure 4D).

2.4. Ce in Plant Fractions

Table 1 presents the Ce concentration in different plant tissues. Statistical analysis showed a significant effect of the factor “species” ($p = 0.0289$ *) (Table S5). Hence, Ce assimilation is also very likely to occur in different ways in *H. lanatus* and *D. tenuifolia*. The Ce concentration measured in the plant roots is relatively low compared to the Ce concentration detected in the substrate after the soil treatment with $n\text{CeO}_2$ (290 mg kg^{-1}).

Considering that the roots were acid washed to remove the fraction attached to the roots' surface, Ce internalization was relatively low (range 3.04–16.1 mg kg⁻¹).

Table 1. Ce concentration ($\mu\text{g kg}^{-1}$ dry weight) in roots, stems and leaves of *H. lanatus* and *D. tenuifolia*. Values are means \pm standard deviation (SD). Between treatments means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$). In mean comparison between species, ns is not significant at $p \leq 0.05$, *, indicate significance at $p \leq 0.05$.

Treatment	Ce Roots		Ce Stems		Ce Leaves	
	<i>H. lanatus</i>	<i>D. tenuifolia</i>	<i>H. lanatus</i>	<i>D. tenuifolia</i>	<i>H. lanatus</i>	<i>D. tenuifolia</i>
Ctrl	822 \pm 241c	305 \pm 197c	456 \pm 127a	225 \pm 180b	625 \pm 231a	145 \pm 158b
nCeO ₂ 25 nm	16,079 \pm 5601a	9392 \pm 2797a	556 \pm 257a	140 \pm 55.7b	165 \pm 191a	99.6 \pm 129b
nCeO ₂ 50 nm	5273 \pm 1926b	3037 \pm 666b	661 \pm 472a	886 \pm 85.4a	496 \pm 298a	1044 \pm 195a
Mean	7391 \pm 7413	4244 \pm 4287 *	558 \pm 290	417 \pm 369 ns	429 \pm 279	430 \pm 482ns

Finally, it should be underlined that post-hoc test evidenced a statistically significant difference between Ce concentration in roots. In particular, in both species, the highest Ce concentration was observed in the plants' roots treated with the smaller nCeO₂ (Table 1).

The Ce translocation towards the aerial part of the plants was not very high. Compared to Ce accumulation in plant roots, data suggest that this element's mobilization within plants had a different sense. Furthermore, plant species behaved differently. In *H. lanatus*, Ce concentration in stems and leaves was not statistically significant at ANOVA, whereas in *D. tenuifolia* they were ($p = 0.0005$ *** and $p = 0.0006$ **, respectively). In sharp contrast to what was observed in roots, the highest Ce concentration was recorded in nCeO₂ 50 nm treatment (Table 1).

2.5. Macro and Micronutrient Plant Uptake

The concentrations of essential macroelements (K, Mg, and P) and microelements (Cu, Fe, Mn, and Zn) in the tissues of *H. lanatus* and *D. tenuifolia* at maturity are respectively shown in Tables 2 and 3: Tables S6–S8 reports the results of the statistical analysis. The monocot-dicot comparison was expected to highlight significant differences in the behaviour of the species. As previously observed, also in this case, the ANOVA showed that in the average of the treatments, the effect of the species factor was always statistically significant ($p \leq 0.001$).

Table 2. Macronutrient concentration (mg kg⁻¹ dry weight) in roots, stems and leaves of *H. lanatus* and *D. tenuifolia*. Values are means \pm standard deviation (SD). Between treatments means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$). In mean comparison between species, ns is not significant at $p \leq 0.05$, *, and *** indicate significance at $p \leq 0.05$, and $p \leq 0.001$.

Treatment	K		Mg		Na		P	
	<i>H. lanatus</i>	<i>D. tenuifolia</i>	<i>H. lanatus</i>	<i>D. tenuifolia</i>	<i>H. lanatus</i>	<i>D. tenuifolia</i>	<i>H. lanatus</i>	<i>D. tenuifolia</i>
	Roots							
Ctrl	5500 \pm 657a	14,777 \pm 743a	633 \pm 81.8a	1302 \pm 87.4a	331 \pm 79.8b	187 \pm 98.7a	1224 \pm 136a	6669 \pm 840a
nCeO ₂ 25 nm	6869 \pm 1352a	13,288 \pm 2805a	588 \pm 35.4ab	1551 \pm 334a	390 \pm 125ab	249 \pm 123a	1465 \pm 125a	5969 \pm 1487a
nCeO ₂ 50 nm	5881 \pm 791a	16,545 \pm 2015a	490 \pm 33.5b	1780 \pm 355a	686 \pm 191a	406 \pm 100a	1363 \pm 108a	7659 \pm 742 a
Mean	6084 \pm 1047	14,870 \pm 2413 ***	570 \pm 79.2	1544 \pm 337 ***	469 \pm 204	281 \pm 129 *	1351 \pm 150	6766 \pm 1230 ***
	Stems							
Ctrl	28,927 \pm 2252a	23,694 \pm 2802a	1032 \pm 110a	1014 \pm 394a	115 \pm 11.7ab	265 \pm 142a	2836 \pm 287a	3431 \pm 737a
nCeO ₂ 25 nm	31,580 \pm 2978a	22,466 \pm 3856a	1114 \pm 10.4a	878 \pm 277a	95.5 \pm 9.12b	318 \pm 29.6a	2524 \pm 167a	2875 \pm 9.76a
nCeO ₂ 50 nm	32,065 \pm 3806a	26,355 \pm 663a	1117 \pm 37.7a	932 \pm 208a	128 \pm 15.2a	519 \pm 267a	2668 \pm 291a	3402 \pm 661a
Mean	30,857 \pm 3041	24,172 \pm 2964 ***	1088 \pm 71.9	941 \pm 194ns	113 \pm 17.8	368 \pm 176 ***	2676 \pm 259	3234 \pm 437 *
	Leaves							
Ctrl	31,314 \pm 2192a	30,115 \pm 4571a	1547 \pm 80.9a	2312 \pm 313a	150 \pm 46.6a	1249 \pm 325a	3671 \pm 462a	3138 \pm 628a
nCeO ₂ 25 nm	32,772 \pm 2096a	22,672 \pm 3176a	1397 \pm 51.9a	2459 \pm 94.6a	81 \pm 16.8a	803 \pm 219a	3335 \pm 401a	2677 \pm 429a
nCeO ₂ 50 nm	31,610 \pm 1854a	30,057 \pm 3631a	1508 \pm 13.2a	2267 \pm 229a	126 \pm 77.6a	1094 \pm 206a	3163 \pm 473a	3719 \pm 388a
Mean	31,899 \pm 1836	27,615 \pm 5106 *	1455 \pm 68.2	2346 \pm 192 ***	119 \pm 55.1	1049 \pm 296 ***	3390 \pm 447	3178 \pm 664ns

Table 3. Macronutrient concentration (mg kg⁻¹ dry weight) in roots, stems and leaves of *H. lanatus* and *D. tenuifolia*. Values are means ± standard deviation (SD). Between treatments means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$). In mean comparison between species, ns is not significant at $p \leq 0.05$, *, and *** indicate significance at $p \leq 0.05$, and $p \leq 0.001$, respectively.

Treatment	K		Mg		Na		P		
	<i>H. lanatus</i>	<i>D. tenuifolia</i>	<i>H. lanatus</i>	<i>D. tenuifolia</i>	<i>H. lanatus</i>	<i>D. tenuifolia</i>	<i>H. lanatus</i>	<i>D. tenuifolia</i>	
	Roots								
Ctrl	7.35 ± 0.27a	7.35 ± 0.27a	138 ± 38.3a	46.3 ± 3.14a	19.1 ± 3.34a	4.21 ± 0.86a	159 ± 15.9a	40.3 ± 5.11b	
nCeO ₂ 25 nm	7.84 ± 2.06a	7.84 ± 2.06a	103 ± 20.9ab	14.3 ± 1.97b	12.3 ± 13.6b	2.59 ± 0.44b	101 ± 13.7b	68 ± 10.1a	
nCeO ₂ 50 nm	7.35 ± 1.25a	7.35 ± 1.25a	60.7 ± 19b	13 ± 3.28b	16.7 ± 2.12ab	4.15 ± 0.51ab	131 ± 0.24ab	53.9 ± 5.38ab	
Mean	7.51 ± 1.24	7.51 ± 1.24	101 ± 41.2	24.5 ± 14.9***	16 ± 3.74	3.65 ± 1.03***	130 ± 27.3	54 ± 13.6***	
	Stems								
Ctrl	2.99 ± 0.66a	2.99 ± 0.66a	30.2 ± 11.3a	23.4 ± 10.3a	69.3 ± 19.3a	4.04 ± 0.98a	51.5 ± 4.55a	30 ± 10.6a	
nCeO ₂ 25 nm	2.97 ± 0.37a	2.97 ± 0.37a	17.7 ± 3.65a	14.3 ± 5.11ab	31.4 ± 11.4a	3.15 ± 0.25a	47.0 ± 3.99a	29.5 ± 8.12a	
nCeO ₂ 50 nm	2.76 ± 0.61a	2.76 ± 0.61a	18.7 ± 1.89a	4.97 ± 1.86b	49.9 ± 15.8a	4.71 ± 0.83a	41.0 ± 11.3a	30.9 ± 17.3a	
Mean	2.91 ± 0.5	2.91 ± 0.5	22.2 ± 8.53	14.2 ± 10.4*	51.1 ± 20.5	3.94 ± 0.92***	46.5 ± 7.85	31.8 ± 10.3*	
	Leaves								
Ctrl	3.56 ± 0.52a	3.56 ± 0.52a	34.5 ± 4.60a	42.5 ± 4.52a	47.3 ± 16.9a	9.96 ± 0.80a	26.8 ± 0.40a	100 ± 26.2a	
nCeO ₂ 25 nm	4.47 ± 0.48a	4.47 ± 0.48a	36.1 ± 3.54a	28.9 ± 5.26a	26.8 ± 9.11a	10.7 ± 0.74a	29.2 ± 6.29a	96.5 ± 22.5a	
nCeO ₂ 50 nm	4.13 ± 0.57a	4.13 ± 0.57a	34.0 ± 2.76a	36.0 ± 6.49a	37.6 ± 8.72a	13.5 ± 4.44a	26.0 ± 5.23a	70.0 ± 22.2a	
Mean	4.06 ± 0.6	4.06 ± 0.6	34.9 ± 3.35	35.8 ± 7.29ns	37.3 ± 13.8	11.4 ± 2.98***	27.3 ± 4.34	88.4 ± 20.9***	

In the root tissues of *D. tenuifolia* the concentration of K, Mg and P was respectively 2.44, 2.71, and 5 times higher than *H. lanatus*. In the case of Na, the situation was the opposite and the greatest concentration of this element was recorded in the roots of *H. lanatus* (+1.67 times compared to *D. tenuifolia*, $p \leq 0.01$) (Table 2). More important concerning the purpose of this study was the effect of the treatments. It was statistically significant only in the roots of *H. lanatus* for Mg and Na. The variation in the concentration of the two elements had an opposite trend. The concentration of Mg decreased ($p = 0.0472$ *) from 633 mg kg⁻¹ (Ctrl) to 490 mg kg⁻¹ (nCeO₂ 50 nm), while Na concentration increased ($p = 0.0458$ *) from 331 mg kg⁻¹ (Ctrl) to 686 mg kg⁻¹ (nCeO₂ 50 nm) (Table 2).

By observing the distribution of macronutrients in the aerial tissues of plants, a statistically significant effect of the factor is confirmed, especially in the case of K, Na and P. As expected, the highest concentration of K was found in *H. lanatus* ($p = 0.0004$ ***), while the greater accumulation of Na ($p = 0.0009$ ***) and P ($p = 0.0198$) was observed in the stems of *D. tenuifolia*. Moreover, the foliar tissues of *H. lanatus* accumulate more K than *D. tenuifolia* ($p = 0.0115$ *), while the dicot species allocates most Mg ($p = 0.0000$ ***). In both species, the aerial fractions do not show statistically significant effects due to the treatments with nCeO₂ (Table 2). The statistically significant effect of the factor "species" was confirmed in the case of microelements. In particular, in the roots of *H. lanatus* the concentrations of Cu, Fe, Mn, and Zn are always higher than in *D. tenuifolia* ($p = 0.0000$ *** for each element) (Table S6). The same evidence is also confirmed in the stems for Cu ($p = 0.0008$ ***), Fe ($p = 0.0289$ *), Mn ($p = 0.0000$ ***) and Zn ($p = 0.0108$ *) (Table S7). Lastly, in the leaf tissues, we observed a higher concentration of Mn in *H. lanatus* ($p = 0.0000$ ***), while Zn accumulates more in the leaves of *D. tenuifolia* ($p = 0.0000$ ***) (Table S8).

Except Cu, the root uptake of micronutrients responded to the nCeO₂ treatments, which determined a statistically significant response to Mn ($p = 0.0441$ *) and Zn ($p = 0.0034$ **) but in both species, no clear trends were recorded. On the contrary, we found an apparent adverse effect for Fe root uptake ($p = 0.0375$). In *H. lanatus*, the plant responds differently to nCeO₂ 25 nm and nCeO₂ 50 nm, which reduces the concentration of Fe of 25% and 56%, respectively. In *D. tenuifolia*, the response to nCeO₂ 25 nm and 50 nm is not statistically different. However, treatment determined a substantial decrease in Fe accumulation (−70.5%) (Table 3). Finally, considering the leaf tissues, only the concentration of Cu in *D. tenuifolia* is affected by the treatments, which determines an increase in the concentration of the element (Table 3).

3. Discussion

This paper demonstrates that the growth of *D. tenuifolia* was not influenced by nCeO₂, while, although nCeO₂ concentration was not very high (200 mg kg⁻¹) compared to several studies among those cited above, *H. lanatus* responded to the treatment.

The most evident effect of the treatments occurred on developing the aerial biomass of *H. lanatus*. This was unexpectedly observed in response to treatment with the $n\text{CeO}_2$ 50 nm. As presented above, the larger nanoparticles stimulated a more significant number of stems per plant. In turn, greater tillering has increased aerial biomass accumulation and, therefore, a greater surface area of photosynthetic tissues was produced. This stimulating effect agrees with other observations made on autumn-spring cereals *T. aestivum* and *H. vulgare* [18,20]. In these experiments, plants were grown in soil amended with $n\text{CeO}_2$ and responded to the highest treatment (500 mg kg^{-1}) with increased plant height and aboveground dry biomass. In addition, the yield components of both species were negatively affected by the treatments. A confirmation of the negative impact of $n\text{CeO}_2$ (at 800 mg kg^{-1}) on grain yield was provided for *Zea mays*, as well [19].

Going back to the effects detected on *H. lanatus*, it must be specified that the observed increase in leaf area did not correspond the enhancement of the dry weight of the leaves as it would have been reasonable to expect. So, the newly formed leaves were thinner than those previously formed. According to similar observations on *H. vulgare* [21], it is likely that treated plants had a longer vegetative period than the controls. Conflicting evidence has been reported on dicotyledonous species exposed to $n\text{CeO}_2$. Positive responses in terms of growth and yield of *L. esculentum* were recorded at low concentrations ($10\text{--}125 \text{ mg kg}^{-1}$) [27] whereas, at higher concentrations, $n\text{CeO}_2$ adversely affect the plant metabolism and growth [38]. The same consequences have been verified in *G. max* [22], *C. sativus* [23] and *L. esculentum* [28].

Like other metal-oxide nanoparticles (e.g., $n\text{CuO}$, $n\text{TiO}_2$, and $n\text{ZnO}$), $n\text{CeO}_2$ has high stability and a low dissolution rate [38]. However, after interactions with plant tissues, nanoparticles may undergo dissolution and aggregation [39]. Aggregation is how the metal nanoparticles lose the monodisperse state and tend to associate in clusters of varying sizes. The process is mainly due to Van der Waals and electrostatic forces in a stock solution depending on the particle size, the surface properties, and charge [40]. The aggregation processes between nanoparticles can also occur in the soil, in the rhizosphere, and within plant tissues. However, the mechanisms of interaction between nanomaterials, possibly endowed with positive or negative charges, and biomolecules have not yet been fully elucidated [41].

The presence of aggregates of $n\text{CeO}_2$ in plant tissues has been previously observed [42,43]. However, it was reported that the particle agglomeration occurs before the passage from roots to the other plant fractions [44]. In this experiment, the increase in the dissolved Ce fraction in roots exposed to $n\text{CeO}_2$ 50 nm could be a proxy of an aggregation process that previously occurred within the root tissues. The aggregation of $n\text{CeO}_2$ taken up by roots and the subsequent release of cerium ions inside roots prevents the translocation of $n\text{CeO}_2$ towards the aerial plant biomass [45,46]. According to [47–49], fewer and smaller in size $n\text{CeO}_2$ have detected the plant leaves. As for particle translocation, it has not conclusively clarified whether $n\text{CeO}_2$ can move via a symplastic or apoplastic pathway [50].

Albeit such a specific aspect was not investigated in this study, the differences we observed can likely be explained at this level. So, we hypothesize that in the rhizosphere of *D. tenuifolia*, much more favorable conditions for the $n\text{CeO}_2$ aggregation exist than in *H. lanatus*. On the other hand, the root morphology of monocots and dicots is very different [51]. The root uptake of nanoparticles is influenced by the specific surface of the root apparatus and the emissions of mucilage compounds and root exudates [44,52].

As for Ce concentration in plant fractions, an increase of Ce concentration in the roots with a linear trend was observed in treated plants while a limited translocation of Ce to the aboveground plant biomass. Our data corroborated those provided by several studies carried out in similar conditions [18–21,23,27,28].

Although $n\text{CeO}_2$ treatments did not affect plant roots' development, given their functional role, it is also essential to verify whether treatments influenced nutrient assimilation. Cerium belongs to the rare earth elements (REE). Due to the increasing industrial exploitation of such elements, they are currently considered emerging pollutants. Their

plant toxicity mechanisms are not completely clear yet. However, a solid demonstration regarding the influence of Ce on plant nutrition was already provided [53,54].

The elemental composition of edible products of crops grown in a solid media treated with $n\text{CeO}_2$ has been previously investigated in monocots [19,20,55–57] and dicots [27,58–60]. These studies evaluated the nutritional value of crop yield eventually influenced by the ENMs. The focus of this work is mainly ecological. However, even in this perspective, it is relevant to check whether plant exposure to $n\text{CeO}_2$ has any consequences on the uptake of macro and microelements, which can be evidence of plant stress and negative consequences on the food web [61].

Considering literature data, we can draw the following summary considerations. First, the concentration of macro and micronutrients in plants varies with soil types; therefore, even from solidly organized experiments, it is not easy to generalize nutritional imbalances in plants exposed to ENM. Second, $n\text{CeO}_2$ influence the assimilation of some elements in the exposed plants. As for macronutrients, the literature provides clear indications of the negative effect of $n\text{CeO}_2$ on the uptake of some elements. Data on *H. lanatus* confirm a significant reduction in Mg uptake due to $n\text{CeO}_2$, already observed in *T. aestivum* [57,60]. As for Na, it was reported a decrease of concentration in barley plants, while we observed an increase of this element in treated *H. lanatus*. Still conflicting indications concern the assimilation of K and P in plants treated with $n\text{CeO}_2$ [20,60], whereas no responses were observed in *H. lanatus* and *D. tenuifolia*.

The root uptake of micronutrients is more sensitive to the presence of $n\text{CeO}_2$ than macronutrients. Therefore, a micronutrient imbalance could be the most critical effect of ENMs on plants. In this study, the clearest example of the influence of $n\text{CeO}_2$ on the assimilation of nutrients concerned Fe. A relevant reduction in root Fe uptake in both *H. lanatus* and *D. tenuifolia* was observed. Moreover, in the dicot, the lower Fe accumulation was observed in the plant stems but not in the leaves. Contrarily, other authors reported a remarkable increase in Fe accumulation in soybean plants [60]. Manganese uptake in *H. lanatus* and *D. tenuifolia* was negatively affected by $n\text{CeO}_2$ 25 nm and, to a lesser extent, by the larger $n\text{CeO}_2$. Similar results were obtained on *Triticum aestivum* [57] but not on *Glycine max* [22]. Finally, the Zn root concentration in treated plants compared to the control decreased in *H. lanatus* and increased in *D. tenuifolia*. Similar contradictory results are provided by the literature [20,60].

4. Materials and Methods

4.1. $n\text{CeO}_2$ Characterization

The cerium oxide nanopowders ($n\text{CeO}_2$) with an average particle size of 25 nm and 50 nm respectively were purchased from Sigma-Aldrich (St. Louis, MO, USA). $n\text{CeO}_2$ have a density of 7.13 g mL^{-1} at $25 \text{ }^\circ\text{C}$ and 99.95% of purity (81.25% of Ce). The suspensions were characterized for hydrodynamic diameter (Hd), Z-average size, relative polydispersity index (PDI), and ζ -potentials. The size and average shape were measured with a transmission electron microscope (TEM, FEI Tecnai F20, FEI Company, Eindhoven, The Netherlands). The $n\text{CeO}_2$ were suspended in deionized water and sonicated in a water bath for 60 min with a sonication intensity of 180 W cm^{-2} . The hydrodynamic diameter (Hd) and the Z-average size on $n\text{CeO}_2$ were measured by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Ltd., Worcestershire, UK). ζ -potentials were quantified by laser Doppler velocimetry as the electrophoretic mobility, using a disposable electrophoretic cell (DTS1061, Malvern Ltd., Worcestershire, UK).

4.2. Experimental Conditions

Seeds of *H. lanatus* were purchased by SemeNostrum (Udine, Italy), while seeds of *D. tenuifolia* were provided by Sementi Bruni (Corbetta, Milan, Italy). The soil used for this experiment was Compo Sana organic potting mix (soil pH = 6.8–7.2). The potting substrates were amended with two water suspensions, one with 25 nm $n\text{CeO}_2$ and the other with 50 nm $n\text{CeO}_2$, in order to reach a final substrate concentration of 200 mg kg^{-1}

$n\text{CeO}_2$. Before this amendment, which occurred in a single dose through irrigation, the suspensions were stirred and sonicated for 30 min to avoid aggregation. Tap water was used as control. After the irrigation, the amended soils were carefully mixed in order to obtain uniformity. Subsequently, the $n\text{CeO}_2$ amended substrates were stored in the dark at 15 °C for three days for conditioning before planting seeds. After equilibration, 500 g of control and amended substrate were put in the pots (Figure S1). The experiment was carried out in a semi-controlled greenhouse facility.

Seeds were sowed about 2.5 cm deep in the soil and pots were placed in full sunlight, at 18–27 °C (night/day) with a relative humidity around 60% (Figure S6). Two weeks after sowing, the seedlings were thinned to two seedlings per pot. An additional set of plants were grown to observe the entry of $n\text{CeO}_2$ into plant tissues. During the trial, pots were irrigated every three days and randomly reallocated every week. During the plant growth the pots were irrigated to maintain the substrate at 60% of water holding capacity (Figures S7 and S8). After 60 days, control and treated plants were harvested. Fresh plant biomass was separated in roots, stems, and leaves and weighed. Then the plant fractions were thoroughly washed in tap water and rinsed three times with distilled water. In addition, roots were washed in 400 mL of 0.01 M of HNO_3 in a shaker bath at 300 rpm for 5 min to remove metal ions adsorbed at the surface. Leaf area was measured using a LI-3100C Area Meter (Li-Cor Corporation, Lincoln, NE, USA). Finally, the plant fractions were oven dried at 60 °C for three days and weighed. Additional information regarding the experimental setup are reported in Supplementary Information.

4.3. Detection of $n\text{CeO}_2$ in Plant Fractions

Before setting up the main factorial experiment, it was carried out a preliminary test with the aim of verifying that in our experimental conditions it would have been possible to trace the $n\text{CeO}_2$ inside the plant tissues. For this reason, other plants were grown under the same conditions as in the main experiment. Twenty days after germination, the uptake and translocation of $n\text{CeO}_2$ in plants was investigated by enzymatic digestion [62]. The digesting enzyme used is Macerozyme R–10 Pectinase from *Rhizopus* sp. (Sigma Aldrich, St. Louis, MO, USA). The technical details are reported in Supplementary Information.

4.4. Cerium Concentration in Plant Tissues

An amount of 0.3 g was sampled from the oven-dried plant fraction and digested on a CEM microwave oven (MARS Xpress, CEM, Matthews, NC, USA), using 9 mL of HNO_3 (65%) and 1 mL of hydrogen peroxide (H_2O_2) in Teflon cylinders at 180 °C, according to the USEPA 3052 method [63]. After mineralization, plant extracts were filtered at room temperature under fume hood with Whatman 0.45 μm PTFE membrane filters, and finally diluted and analyzed. Determinations of the Ce total content were carried out using a ICP-MS NexION 350 (PerkinElmer, Waltham, MA, USA). The accuracy of the analytical procedure adopted for ICP-MS analysis was checked by running standard solutions every 20 samples. Yttrium was used as the internal standard.

4.5. Calculations and Statistical Analysis

The trial was set up in a randomized experimental design with the following factors: (i) two plant species, (ii) Ce concentration: 0 (Ctrl) and 200 mg kg^{-1} $n\text{CeO}_2$, (iii) $n\text{CeO}_2$ particle size: 25 nm and 50 nm, respectively. Each treatment was replicated four times. From the dry weight of plant fractions and leaf area, the following ratios were calculated: root to shoot ratio (R/S ratio), the fraction of total plant biomass allocated to roots (RMF, root mass fraction), and stems (SMF, stem mass fraction), and the leaf area ratio (LAR) (Table S3). Statistical analysis was carried out with one-, two- and three-way ANOVA. When necessary, data were subjected to logarithmic transformation prior to analysis, which effectively homogenized the variances and produced approximately normal distributions. Statistically significant differences correspond to $p \leq 0.05$. In case of significant effects, Tukey's Multiple Comparison test was used to analyze individual effects. Different letters

in tables and graphics are used to indicate means that are statistically different. Single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) data on $n\text{CeO}_2$ size distribution were processed by means of Syngistix Nano Application Module software (Perkin Elmer, Waltham, MA, USA), and interpolated with Gaussian curves.

5. Conclusions

Literature shows that the most studied terrestrial plant species are radish, rape, ryegrass, lettuce, corn, and cucumber. However, the documented and modeled flows of ENMs in the environment also affect wild plants. Such plants live in uncultivated natural areas and contribute to supporting the food web and ecosystem services (e.g., C-cycle, interactions with microbes and insects).

In this study, the first question concerned the uptake and translocation of $n\text{CeO}_2$ within the studied plant species. Plant roots indeed assimilated $n\text{CeO}_2$ with a magnitude depending on their size; however, only a small fraction of them moved through to the aerial plant parts. A difference in $n\text{CeO}_2$ assimilation due to the species was observed, as well. A second aspect we studied concerns the effects on plant growth. During the growth cycle, the monocot species benefited from the treatment with $n\text{CeO}_2$ 50 nm by accumulating more biomass in the aerial fractions and increasing the photosynthesizing surface. However, it must be emphasized that the concentration of $n\text{CeO}_2$ we used is not very high, being comprised—according to the literature—in the range that stimulates plant growth. This likely hidden the expected $n\text{CeO}_2$ size-effects, whereas, at higher concentrations, we might have appreciated this effect. However, even in the absence of evident negative anomalies on plant growth, we observed alterations in macro and micronutrients' root uptake.

Whether early studies on ENMs' plant relationships regarded punctual observations on plants exposed to high doses of ENMs, current investigations are currently developing more and more intensely in different directions. Some examples are (i) effects of ENMs low doses over time, (ii) ENMs biotransformation, and (iii) the omics data describing the complex plant molecular networks in ENMs response. However, from an ecological perspective, it is of fundamental importance to develop adequate studies on spontaneous species. However, the impact of these materials on food chains has not yet been adequately studied.

In general, wild plant species have high plasticity by combining both the long-term accumulation of genetic changes and conserving survival strategies through time. On the other hand, the requested high productive performances of crops are often genetically obtained to the detriment of biotic and abiotic stresses' adaptation ability. So, it seems plausible to hypothesize that the ENMs-plants relationships may be different in wild species than cultivated ones, also keeping into account that they are possibly exposed to ENMs fluxes for a more extended time. These aspects deserve adequate attention and will undoubtedly open new investigation fields in understanding the consequences ENMs fluxes in the ecosystems.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2223-7747/10/2/335/s1>, Figure S1: Preparation of the experiment: filling the pots with the $n\text{CeO}_2$ amended substrate. Figure S2: Plantlets of *H. lanatus* and *D. tenuifolia* 10 d after sowing. Figure S3: Plants of *H. lanatus* and *D. tenuifolia* 30 day after sowing. Figure S4: Plants of *H. lanatus* (in the background) and *D. tenuifolia* before biomass harvesting. Figure S5: Stems dry matter \pm standard deviation of *H. lanatus* and *D. tenuifolia*. Comparison between control and plants grown in presence of 200 mg kg^{-1} $n\text{CeO}_2$ having respectively 25 nm and 50 nm. For each species the statistically significant difference ($p \leq 0.05$) between treatments is indicated by the letters using one-way ANOVA followed by Tukey's test. Figure S6: Total plant dry matter \pm standard deviation of *H. lanatus* and *D. tenuifolia*. Comparison between control and plants grown in presence of 200 mg kg^{-1} $n\text{CeO}_2$ having respectively 25 nm and 50 nm. For each species the statistically significant difference ($p \leq 0.05$) between treatments is indicated by the letters using one-way ANOVA followed by Tukey's test. Figure S7: Leaf mass fraction \pm standard deviation of *H. lanatus* and *D. tenuifolia*. Comparison between control and plants grown in presence of 200 mg kg^{-1} $n\text{CeO}_2$ having respectively 25 nm and 50 nm. For each species

the statistically significant difference ($p \leq 0.05$) between treatments is indicated by the letters using one-way ANOVA followed by Tukey's test. Figure S8: Specific leaf area* \pm standard deviation of *H. lanatus* and *D. tenuifolia*. Comparison between control and plants grown in presence of 200 mg kg⁻¹ nCeO₂ having respectively 25 nm and 50 nm. For each species the statistically significant difference ($p \leq 0.05$) between treatments is indicated by the letters using one-way ANOVA followed by Tukey's test. * According to Evans (1972) SLA is the total leaf area of a plant divided by the total leaf weight. This ratio has a relevant ecological importance as describes the allocation of leaf biomass relative to leaf area which in turns refers to carbon gain relative to water loss, within a plant canopy (Gunn et al., 1999). Table S1—Most frequent particle size, mean particle size, number of peaks and content of dissolved Ce determined by sp-ICP-MS analysis after enzymatic extraction on roots and leaves of *H. lanatus* and *D. tenuifolia* treated with nCeO₂ 200 mg L⁻¹. Table S2—Two-way ANOVA p value determined for biometric variables of *H. lanatus* and *D. tenuifolia*. ns is not significant at $p \leq 0.05$, *, ** and *** indicate significance at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Table S3—Biomass allocation variables calculated from plant measurements (Porter et al., 2011). Table S4—Two-way ANOVA p value determined for biometric ratios calculated for *H. lanatus* and *D. tenuifolia*. ns is not significant at $p \leq 0.05$, *, ** and *** indicate significance at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Table S5—Two-way ANOVA p value determined for Ce concentration in plant fractions of *H. lanatus* and *D. tenuifolia*. ns is not significant at $p \leq 0.05$, *, ** and *** indicate significance at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Table S6—Two-way ANOVA p value for concentration of macronutrients and micronutrients in roots of *H. lanatus* and *D. tenuifolia*. ns is not significant at $p \leq 0.05$, *, ** and *** indicate significance at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Table S7—Two-way ANOVA p value for concentration of macronutrients and micronutrients in stems of *H. lanatus* and *D. tenuifolia*. ns is not significant at $p \leq 0.05$, *, ** and *** indicate significance at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Table S8—Two-way ANOVA p value for concentration of macronutrients and micronutrients in leaves of *H. lanatus* and *D. tenuifolia*. ns is not significant at $p \leq 0.05$, *, ** and *** indicate significance at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

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