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1 **Design defines the effects of nanoceria at a low dose on soil microbiota and the potentiation of**
2 **impacts by canola plant**

3

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28

29 Abstract:

30 Soils act as nanoceria sinks *via* agricultural spreading and surface waters. Canola plants were grown
31 for one month in soil spiked with nanoceria (1 mg.kg⁻¹). To define the role of nanomaterials design on
32 environmental impacts, we studied nanoceria with different sizes (3.5 or 31 nm) and coating (citrate).
33 We measured microbial activities involved in C, N and P cycling in the rhizosphere and unplanted
34 soil. Bacterial community structure was analyzed in unplanted soil, rhizosphere and plant roots by
35 454-pyrosequencing of the 16S rRNA gene. This revealed an impact gradient dependent on
36 nanomaterials design, ranging from decreased microbial enzymatic activities in planted soil to
37 alterations in bacterial community structure in roots. Particle size/aggregation was a key parameter in
38 modulating nanoceria effects on root communities. Citrate coating lowered the impact on microbial
39 enzymatic activities but triggered variability in the bacterial community structure near the plant root.
40 Some nanoceria favored taxa whose closest relatives are hydrocarbon-degrading bacteria, and
41 disadvantaged taxa frequently associated in consortia with disease-suppressive activity toward plant
42 pathogens. This work provides a basis to determine outcomes of nanoceria in soil, at a dose close to
43 predicted environmental concentrations, and to design them to minimize these impacts.

44

45 Introduction

46 Cerium oxide-based nanoparticles (nanoceria) have acquired great interest for their oxygen storage
47 capacities, as well as their optical and catalytic properties. With a global estimated production of
48 10,000 metric tons/year.^{1,2} nanoceria are used in a variety of industrial sectors including fuel cells,
49 electronic and optical devices, chemical mechanical polishing, exterior paints, and diesel fuel
50 additives.³ Some of these applications, such as coatings and paints, are dispersive during use phase.
51 Modeling of the environmental exposure to engineered nanomaterials (ENMs) shows that soils are
52 critical sinks for ENMs.⁴ Keller and Lazareva estimated the global emission of nanoceria in soil to be
53 in the range of 129-1029 metric tons/year.⁵ This elevated value is certainly a concern for the potential
54 risks of ENMs on soil.

55 Soil microbial communities provide critical ecosystem services including nutrient mineralization and
56 cycling, and contribute to plant growth and health.⁶ ENMs added to soil can have adverse effects on

57 microorganisms. In microcosms, nanoTiO₂ and nanoZnO (0.5-2 g.kg⁻¹ soil) reduce microbial biomass
58 and diversity and alter the composition of soil bacterial communities.⁷ NanoTiO₂ transiently decreases
59 C mineralization at 1 mg.kg⁻¹ in silty soil rich in organic matter.⁸ Nanosized particles of copper oxide
60 (CuO) and magnetite (Fe₃O₄) (<50 nm, 1 g.kg⁻¹) change the hydrolytic activity and bacterial
61 community composition of sandy clay loam soil⁹ Colman et al. demonstrated the reduction of
62 microbial extracellular enzymes and microbial biomass in soil treated with nanosilver (0.14 mg of Ag
63 kg⁻¹ soil) in a mesocosm study.¹⁰ Recently, Ge et al. showed that nanoceria (100 mg.kg⁻¹) alter soil
64 bacterial communities in soil planted with soybean,¹¹ although they do not affect soil bacterial
65 communities in unplanted soils. The authors suggest that plants interactively promote nanoceria
66 effects in soil, probably due to belowground carbon changes that result from toxic impact and plant
67 growth decrease.

68 The interactions between plant roots and soil create a narrow zone of soil known as the rhizosphere,
69 which is considered to be one of the most dynamic interfaces on earth.¹² Numerous biogeochemical
70 processes take place at the plant root-soil interface, which are driven by plant root activity and
71 mediated by soil microorganisms. These processes regulate terrestrial carbon and other cycling
72 elements that sustain plant growth, as well as food, fuel and fiber production.¹³ Understanding how
73 ENMs can interact within the rhizosphere to influence plant and microbial community function and
74 structure is thus crucial for a variety of ecosystem level processes. However, current knowledge on the
75 impact of ENMs on plants and the rhizosphere microbiota is limited.

76 To date, research on the environmental impact of ENMs on soil has largely focused on high
77 concentrations of nanomaterials; by contrast, soil environmental concentrations of nanoceria are
78 predicted^{14, 15} within the range of 0.28-1.12 mg.kg⁻¹ and could be even lower (0.09 to 5.1mg.kg⁻¹) as
79 predicted in Denmark.¹⁶ The current study therefore investigates the impact of nanoceria on a soil-
80 plant-bacteria micro-ecosystem at a concentration of 1 mg.kg⁻¹, which is close to an environmentally
81 relevant nanoceria concentration.

82 Previous studies have highlighted the influence of physicochemical factors such as geometry, coating
83 and surface functional groups on the biological effects of nanoparticles.^{17, 18} Nevertheless, the
84 consequences of nanomaterials design still need to be examined in soil. In the case of nanoceria,

85 particle diameter is critical regarding surface defects, $\text{Ce}^{3+}/\text{Ce}^{4+}$ ratio, and a series of properties such as
86 O_2 storage, enzymatic-mimetic activities.¹⁹ Here, we have evaluated the influence of nanomaterials
87 design on soil bacterial microbiota. The design criteria were focused on particle size and surface
88 charge using pristine or citrate-coated nano- CeO_2 , with different average particle diameters and
89 surface coating.

90 The microbial activities involved in C, N and P recycling were examined in unplanted soil, and soil
91 planted with canola after one month of exposure to nanoceria. Microbial community structure was
92 analyzed by 454 pyrosequencing of the *rrs* gene encoding 16SrRNA in planted and unplanted soils,
93 and in plant root systems. Our results reveal a range of gradual impacts dependent on nanomaterials
94 design, from decreased microbial enzymatic activities to alterations in bacterial community structure.
95 More importantly, some nanoceria could exert a selection pressure favoring taxa those closest relatives
96 are aromatic hydrocarbon-degraders, more likely to be resistant to heavy metals and antibiotics, as
97 well as disadvantaged taxa often identified in consortia associated with soil suppressiveness toward
98 plant pathogens. Our results thus provide a basis to determine the potential outcomes of nanoceria in
99 soil, and to design them so as to minimize these impacts, at realistic environmental concentration.

100

101 **Materials and Methods**

102 **Characteristics of CeO_2 nanoparticles**

103 Three different types of commercially available CeO_2 nanoparticles (NPs) were added to the soil. Two
104 types were commercial crystallites of cerianite, with sizes of 31 ± 18 nm, as measured by transmission
105 electron microscopy (TEM) (CeO_2 -U; Nanograin® Umicore), and 3.5 ± 0.5 nm (for CeO_2 -R). Citrate-
106 coated crystallites of CeO_2 -R are used as UV-stabilizer and have a TEM size of 3.9 ± 1.8 nm (coated
107 CeO_2 ; Nanobyk®-3810, Byk). These NPs have been previously described²⁰⁻²² and their characteristics
108 are summarized in Supporting Information (SI) Table S1. CeO_2 NP suspensions were prepared in
109 ultrapure water (UPW; Milli-Q®, Millipore). The compared colloidal behavior of CeO_2 -NPs in a
110 natural mineral water (pH 7.9), in interaction with clays (kaolinite), and the same natural water in
111 aquatic mesocosms (organic carbon 2.0 ± 0.1 $\text{mg}\cdot\text{L}^{-1}$) is fully described.²⁰⁻²² Briefly, pristine CeO_2 -U
112 tends to rapidly homo-aggregate in UPW (Table S1) and in mineral water (with or without clays),²²

113 and is referred to as pristine CeO₂ aggregate in the text. Homo-aggregation in conjunction with hetero-
114 aggregation with clays was observed for pristine CeO₂-R. Citrate-coated CeO₂-NPs homo- and hetero-
115 aggregated but this required time-dependent degradation of the coating.

116

117 **Soil**

118 Topsoil (0–20 cm) from a clay-loam calcareous soil (pH 8.19) was collected from an agricultural
119 parcel used for wheat culture in Aix-les-Platanes (France; 43°33'45.58'' N; 05°28'38.78'' E). The soil
120 was sieved to 4 mm and stored at room temperature before use. Water pH of the soil was 8.19, with
121 4.3% organic matter, 152 mequiv.kg⁻¹ cation exchange capacity, 27.1% sand, 21.5% silt, and 18.9%
122 clay (Gammsol; InVivo Labs, France). Additional soil characteristics are summarized in Table S2.

123 Briefly, 1 kg of soil was deposited in a thin layer in a plastic bag. A suspension of CeO₂-NPs was
124 distributed on the surface to achieve 1.0 mg.kg⁻¹ dry soil mass and a humidity of 15% (w/w). The
125 control soil was treated identically, but without the addition of NPs. The bag was closed and the soil
126 was homogenized by repeatedly inverting the bag. Soil was sieved again to 4 mm. Sterile syringes (60
127 mL) were used as pots and filled with 70 g of soil. Three replicates were prepared for each treatment
128 (control, CeO₂-R, CeO₂-U, and coated CeO₂).

129

130 **Plant growth**

131 Canola seeds were planted and plants were grown in phytotrons (SI). Plants were harvested after 30
132 days of growth. Aboveground plant parts were cut and weighed (fresh weight), dried at 60°C for three
133 days, and weighed again (dried weight). The root system was retrieved from planted pots and washed
134 repeatedly with sterile UPW. Excess water was blotted on sterile tissue paper and the root system was
135 frozen in liquid N₂ and stored at -80°C or alternatively dried at 60°C for three days (dry root mass).
136 The entirety of soil from planted pots was considered as rhizosphere,¹¹ which was manually cleared
137 from root material, collected, frozen in liquid N₂ and stored at -80°C. Soil from unplanted pots,
138 considered as bulk soil, was collected in the same way.

139

140 **Enzymatic activity of soil and catalase mimetic activity of cerium oxide nanoparticles**

141 Soil enzyme activities were determined for fresh collected materials. All enzyme activities were
142 performed in triplicate for each treatment. Enzymatic activities are reported as the mean of the three
143 determinations expressed in unit activity per g of dry soil mass. (SI).

144 Catalase mimetic activity of nanoceria was measured by the decrease in the absorbance of H₂O₂ at
145 240 nm, using a UV–visible spectrophotometer as described in Singh and Singh.²³

146

147 **DNA extraction and quantification and quantification**

148 Total DNA was extracted from 0.5 g of soil or a root fraction using the FastDNA™ SPIN Kit for Soil
149 and FastPrep®-24 Instrument (MP Biomedicals; Illkirch, France), according to the manufacturer's
150 instructions. DNA concentration was determined by spectrophotometry (NanoVue™-NV-GE,
151 Healthcare Limited; UK), which measured the absorbance of the samples at 260-280 nm. As a sample
152 control, fragments of the universal *rrs* gene (encoding 16S rRNA) were amplified by PCR using the
153 universal bacterial primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3'; positions 8-27 of the *rrs*
154 gene from *E. coli*) and S17 (5'-GTTACCTTGTTACGACTT-3'; positions 1492-1509 of the *rrs* gene
155 from *E. coli*). The products were analyzed by electrophoresis in 1.5% (w/v) agarose gels.

156 Total bacteria abundance was measured in the soil by quantitative PCR targeting of *rrs*. (SI).

157

158 **Sequencing and post-run analysis**

159 Barcoded amplicon sequencing processes (bTEFAP®) were performed by MR DNA
160 (www.mrdnalab.com, MR DNA; Shallowater, TX) and are described in Dowd et al.²⁴ The *rrs* gene
161 universal eubacterial primers 27Fmod (5'-AGRGTTTGATCMTGGCTCAG-3') and 530R
162 (CCGCNGCNGCTGGCAC) were used to amplify the *rrs* gene regions V1–V3. Further details are
163 provided in SI.

164 Sequence data resulting from the sequencing process were processed using a proprietary analysis
165 pipeline (www.mrdnalab.com, MR DNA; Shallowater, TX). Analysis of high-throughput community
166 sequencing data was performed with QIIME version 1.8.²⁵ Further details are provided in SI. A total
167 of 459,709 valid reads and 51,185 OTUs were obtained from the 36 samples through 454
168 pyrosequencing analysis. These OTUs were assigned to 29 different phyla. Each of the 36

169 communities contained between 3,285 and 35,089 reads, with OTUs ranging from 718 to 8,430 reads.
170 The rarefaction curves (consisting of observed OTUs and the Chao1 estimator) tended to approach the
171 saturation plateau for roots, but not for rhizosphere and bulk soil samples (Fig. 1a). Good's coverage
172 estimations revealed that 86-95% of the species were obtained in root samples, whereas 82-88% of the
173 species were determined in soil samples. Although no OTU-level rarefaction curves plateaued under
174 the current sequencing depth, the Shannon diversity indices reached stable values (Figure S1b). This
175 suggests that most of the microbial dominant diversity has been addressed in this data set.²⁶

176

177 **Statistical Analyses**

178 The variables were checked for normality (Shapiro-Wilk test) and homoscedasticity (Levene test). The
179 significance of results was examined by one-way analysis of variance (ANOVA) followed by post-hoc
180 tests (Student-Newman-Keul test). Pearson correlation coefficients were calculated to measure the
181 strength of the association between microbial enzymatic activities. These analyses were performed in
182 STATGRAPHICS Centurion XVI.II. $P < 0.05$ was considered to be statistically significant.

183 For data that were not normally distributed, permutations (PERMANOVA, 999 permutations) and
184 other non-parametric tests (non-parametric t-tests, Kruskal-Wallis test) were performed in R
185 (<http://www.R-project.org>) or STATGRAPHICS. VEGAN's 'adonis' function was used to perform
186 PERMANOVA with constrain permutations of nested factor ("strata" parameters). For multiple
187 testing, P-values were corrected to Q-values using the Benjamini-Hochberg False Discovery Rate
188 (FDR).²⁷ We selected a non-stringent Q-value set at 0.25 to avoid missing any important leads
189 (<http://www.biostathandbook.com/multiplecomparisons.html>). Parametric and non-parametric
190 statistics were implemented to identify OTU taxonomic groups (community modules) and individual
191 OTUs (community members) that differ quantitatively between treatments within a compartment.²⁸

192 For analyses of OTU taxonomic groups, we prepared abundance matrices at the phylum rank
193 containing the sum of OTU abundances for all OTUs per given taxon. Statistical comparisons of
194 taxonomic groups were made for relative abundance using the Kruskal-Wallis test. OTU richness in
195 soil is the result of a large number of low-count OTUs.²⁸ For individual OTU statistics, the follow-up
196 analysis focused on the abundant community members (ACM)²⁸ defined here as OTUs that account

197 for 0.1% of the total observation (sequence) count of an OTU among the 36 treatments (260,175
198 sequences), representing 56.6% of the initial total count. The ACM matrix, including bulk soil,
199 rhizosphere, and root samples, was rarefied at 1,067 reads and was represented by 99 bacterial OTUs
200 and 38,412 observations. Statistical analysis (ANOVA) was applied to count of bacterial taxa at
201 multiple taxonomic levels.

202

203 **Results**

204 *Nanoceria affect the microbial enzyme activities in the rhizosphere*

205 We measured the activity of hydrolase and oxidoreductase soil enzymes known to be involved in the
206 degradation of a range of substrates that are common constituents of organic matter.²⁹ Keystone
207 enzymes were selected to represent carbon cycling, such as β -glucosidase (polysaccharides, *e.g.*
208 cellulose), peroxidase and phenoloxidase (aromatic carbon, *e.g.* lignin), as well as nitrogen cycling,
209 including endoprotease (peptide, *e.g.* protein) and organic P such as acid and alkaline phosphatases (P
210 monoesters). We also measured catalase activity related to aerobic metabolism. Table 1 summarizes
211 the enzymatic activities of C, N, and P recycling in bulk soil and rhizosphere.

212 In bulk soil, the three types of CeO₂-NPs did not alter any of the enzymatic activities tested (as
213 compared to controls), with the exception of bare CeO₂-U NPs, which significantly decreased catalase
214 (by 7%; $P < 0.05$). In the rhizosphere, all CeO₂-NPs significantly decreased the catalase activity and in
215 a similar extent as compared to controls (by $19 \pm 8\%$, $17 \pm 3\%$ and $22 \pm 2\%$ for pristine CeO₂-R, CeO₂-U
216 and coated CeO₂, respectively).

217 In the rhizosphere, small-size and aggregated pristine CeO₂-NPs both decreased β -glucosidase and
218 alkaline phosphatase activities with similar magnitudes (approximately 19-20%). The three nanoceria
219 types did not affect any of the other assayed enzyme activities (*i.e.* protease, acid phosphatase,
220 peroxidase and phenoloxidase). Some enzymatic activities, such as catalase (for all NPs) and alkaline
221 phosphatase (for pristine CeO₂-R and CeO₂-U), were substantially decreased in the rhizosphere to
222 levels observed in bulk soil. Coated CeO₂-NPs displayed the lowest effects on enzymatic activities,
223 which included decreased catalase activity in the rhizosphere and the absence of any effect on the
224 activity of the other assayed enzymes.

225 Both small-size CeO₂-R and coated CeO₂ exhibited catalase mimetic activity, whereas large-size bare
226 CeO₂-U did not significantly reduce hydrogen peroxide activity (Fig. S2).

227

228 ***Nanoceria do not impact bacterial abundance or richness in the three compartments***

229 Nanoceria did not significantly decrease the microbial biomass based on extractable DNA ($P>0.05$) in
230 soil or the bacterial abundance ($P>0.05$), as revealed by the quantitative PCR data (Table S3).

231 We used alpha diversity to describe the total diversity of OTUs within the different treatment
232 communities (CeO₂-R, CeO₂-U, coated CeO₂ and control) among the three compartments (bulk soil,
233 rhizosphere and root). Alpha diversity was summarized, taking into account measures of richness
234 based on qualitative Chao1 and quantitative Shannon estimators, as well as phylogeny-based measures
235 such as Faith's PD. Figure S2 shows rarefaction curves for OTUs based on observed OTUs and
236 distance box plots for Chao1, Shannon and PD estimators. The Chao1, Shannon index, and PD
237 measures revealed that none of the CeO₂ NP treatments affected the alpha diversity of the microbiome
238 (as compared to the controls) in the root compartment, the rhizosphere or bulk soil, when controlling
239 for compartment status. A significant increase was observed for PD in the bulk soil microbiota of the
240 CeO₂-U treatment as compared to CeO₂-R (Fig. 2; $P=0.014$ and $P=0.024$ in observed OTUs and PD,
241 non-parametric two-sample t test).

242

243 ***Nanoceria greatly affect the root microbiota***

244 In order to identify nanoceria treatments associated with compositional dissimilarity in microbiota, we
245 examined the β -diversity, which is a measure of diversity between samples. Principal coordinates
246 analysis (PCoA; Fig. 1a) and PERMANOVA analysis of unweighted UniFrac distances revealed
247 clustering of microbial communities based on the soil compartment factor ($P=0.0001$), but not on
248 nanoceria treatment ($P=0.32$). Taking into account the relative abundance of organisms, PCoA of
249 weighted UniFrac (Fig. 1b) and Bray-Curtis (Fig. 1c) distances showed clustering based on
250 compartment ($P=0.0001$ and $P=0.001$, respectively; PERMANOVA) but not on treatment ($P=0.15$ and
251 $P=0.16$, respectively). Jackknifed PCoA analysis of unweighted and weighted UniFrac and Bray-
252 Curtis distance metrics resulted in similar conclusions (Fig. S3).

253 Since the “compartment” factor appeared as a main variable causing change in the bacterial
254 microbiota, we assessed the effect of nanoceria treatment within the compartment subgroups.
255 Constrained permutations within compartments showed clustering of microbiota based on treatment
256 for the weighted UniFrac distance ($P=0.009$) and Bray-Curtis distance ($P=0.0129$) but not the
257 unweighted UniFrac distance ($P=0.08$).

258 PERMANOVA analysis of UniFrac distance matrices revealed that $\text{CeO}_2\text{-U}$ induced significant
259 differences in roots, when controlling for compartment status (Table S4). Taking into account
260 compartment status, $\text{CeO}_2\text{-U}$ was observed to induce significant changes in root bacterial microbiota,
261 as compared to both the control ($P=0.002$) and $\text{CeO}_2\text{-R}$ ($P=0.005$), based on weighted UniFrac
262 distances., and significant changes in root bacterial microbiota, in comparison to the control ($P=0.042$)
263 and coated CeO_2 ($P=0.022$), when using unweighted UniFrac distances (Table S4). Regarding the
264 analysis of the weighted or unweighted UniFrac distances, nanoceria treatments did not affect the
265 dominant microbiota in bulk soil or in the rhizosphere (as compared to the control; data not shown).
266 As the rarefaction curve did not plateau, we may miss differences that exist in the rare biosphere,
267 which is currently not quantifiable.²⁸

268

269 *Nanoceria significantly modulate the relative abundance of bacterial taxa in the rhizosphere*

270 Figure 2 displays the bacterial microbiota composition by phylum in the root compartment. Statistical
271 analysis (Kruskal-Wallis) exposed significant differences in the microbiota composition at the phylum
272 level for the different treatments, when controlling for compartment status (Table S5). In roots,
273 significant differences in the relative abundance of bacterial taxa clustered $\text{CeO}_2\text{-U}$ from control
274 treatment. The abundance of the phyla *Fibrobacteres*, *Chloroflexi* and *Proteobacteria* significantly
275 increased in the $\text{CeO}_2\text{-U}$ treatment (0.16%, 1.2% and 64.1%, respectively) in comparison to the
276 control (0.02%, 0.66% and 42.8%, respectively), whereas the abundance of the *Actinobacteria* phylum
277 decreased in the $\text{CeO}_2\text{-U}$ treatment (6.3%), as compared to the control (37.4%).

278 In the root compartment, three phyla differentiated the bacterial microbiota of $\text{CeO}_2\text{-U}$ as compared to
279 $\text{CeO}_2\text{-R}$, with a decrease in *Actinobacteria* (from 21% to 6.3%, $P=0.049$, $\text{FDR}=0.23$), an increase in

280 *Chloroflexi* (0.63-1.2%, P=0.049) and *Proteobacteria* (54.6- 64.1%, P=0.049), and an increase in the
281 FDR threshold (0.23).

282 The phylum *Armatimonadetes* and candidate phylum TM7 were significantly decreased in the CeO₂-R
283 treatment as compared to the control (0.16-0.01%, P=0.046 and 0.75-0.32%, P=0.049, respectively),
284 whereas a compensatory increase was observed in *Proteobacteria* and *Verruimicrobia* (42.7-54.6%,
285 P=0.049 and 1.6-2.3%, P=0.049, FDR=0.17). No significant differences were found in bulk soil or in
286 the rhizosphere compartment (P-value <0.05 although Q value >0.25, data not shown).

287 OTU richness in soil is the result of a large number of low-count OTUs.²⁸ Follow-up analysis focused
288 on the community members that accounted for more than 0.1% of the total OTU counts in the matrix
289 rarefied to 1,067 sequences. Certainly, conclusions based on the abundant community members do not
290 account for the contribution of low-abundance taxa. These rare biosphere taxa have important
291 ecological roles, as reservoirs of genetic and functional diversity, in the resilience of ecosystems.³⁰

292 Statistical analysis (one-way ANOVA) revealed significant differences in the relative abundance of
293 bacterial taxa at multiple taxonomic levels in the root compartment (Table S6). Among all treatments,
294 some OTUs discriminated CeO₂-U from controls and CeO₂-R treatment.

295 In CeO₂-U vs. control, microbiota data revealed a severe decrease in the mean relative abundance of
296 *Janthinobacterium*, *Kribbella*, *Micrococcaceae*, *Streptomyces* and *Pseudoxanthomonas*, and an
297 increase in *Acidovorax*, *Pelomonas* and *Methylibium*. In CeO₂-U vs. CeO₂-R, mean relative
298 abundances were reduced for *Janthinobacterium*, *Micrococcaceae* and *Variovorax*, whereas they were
299 increased in *Acidovorax* and *Methylibium*.

300 *Acidovorax* and *Pelomonas* were barely detected in bulk soil (<0.001%) and the rhizosphere
301 (0.006%)³¹, although they were highly enriched in the root compartment of the CeO₂-U treatment. In
302 CeO₂-U treatment vs. control, the OTUs affiliated to *Acidovorax* accounted for 5.65% (vs. 0.22%),
303 *Pelomonas* for 7.65 % (vs. 1.69%) and *Methylibium* for 38.33% (vs 7.6%). *Methylibium* was the most
304 abundant genus that could discriminate CeO₂-U treatment from the control and CeO₂-R, with an 80-
305 fold enrichment from the bulk soil compartment to the root compartment.

306

307 ***Addition of nanoceria to soil at 1 mg.kg⁻¹ does not impact plant growth***

308 Canola plants were grown from seeds for four weeks in a clay-loam calcareous soil enhanced with
309 pristine (CeO₂-R or CeO₂-U) or citrate-coated CeO₂-NPs at a concentration of 1mg/kg (dry soil mass).
310 Plant growth was monitored by measuring the dry biomasses of below and aboveground plant parts
311 (Fig. S5). None of the CeO₂ NP treatments affected plant growth (one-way ANOVA; P=0.85 for
312 shoots and P=0.55 for roots).

313

314 Discussion

315 *Nanoceria trigger functional differences that do not correlate with changes in bacterial microbiota* 316 *composition*

317 Soil enzymatic activities are recognized sensors of natural and anthropogenic disturbances occurring
318 in the soil ecosystem. They play a crucial role in nutrient cycling and in organic matter decomposition.
319 Furthermore, any dysfunction in the enzymatic activity of soils may disturb the biological equilibrium
320 of soil, which may have ecological and economic consequences.³²

321 In the current study, the addition of CeO₂-NPs to soil was found to lower several hydrolytic and
322 oxidoreductive soil microbial activities. The three different NPs reduced catalase activity in the
323 rhizosphere, with the largest pristine CeO₂-U NPs extending this effect into bulk soil. Catalase activity
324 may be related to the metabolic activity of aerobic organisms and has been used as an indicator of soil
325 fertility.³³ The decrease in catalase activity resulting from the NP treatments suggests an inhibition of
326 aerobic bacterial microbiota. The intrinsic catalase mimetic activity of CeO₂-R and coated CeO₂-NPs
327 could minimize the decrease in the measured enzymatic activity. In the rhizosphere, as compared to
328 controls, pristine small size CeO₂-R and large size CeO₂-U decreased the β-glucosidase activity, which
329 is key in the last limiting step of cellulose degradation (C cycle), and reduced the alkaline phosphatase
330 activity, which is crucial in organic P transformation.

331

332 Based on the quantitative Shannon and qualitative Chao1 richness estimators and the divergence-
333 based PD values did not reflect any impact from CeO₂ NP treatments on bacterial microbiota richness
334 in the bulk soil and rhizosphere compartments, as compared to the controls. Based on UniFrac metrics,
335 which calculate a distance measure between communities using phylogenetic information,³⁴ the

336 diversities observed in bulk soil and the rhizosphere were comparable between NP treatments and
337 controls. Based on the quantification of 16S rRNA gene copies (Table S3), NP treatments did not alter
338 the size of bacterial communities. Many studies have shown some impact of NPs on soil enzymatic
339 activities, often on microbial biomass and diversity. For example, Wang et al. demonstrated that soil
340 catalase activity was significantly decreased by nano-Fe₃O₄ as well as urease activity.³⁵ Fe₂O₃ and
341 ZnO NPs can effectively maintain various soil microbiological processes at 100 mg.kg⁻¹, however
342 higher concentrations (e.g. 500-1000 mg.kg⁻¹) have negative impacts on soil ecology.³⁶ Accordingly,
343 soil protease, catalase and peroxidase activities were inhibited in the presence of the TiO₂ and ZnO
344 nanoparticles.³⁷ Moreover, CuO NPs added to a sandy loam (at 1 and 10 mg.kg⁻¹) had a strong effect
345 on bacterial hydrolytic activity.⁹ These studies were conducted on a time-scale comparable^{35,36} or on
346 much longer periods of time^{9,37}, however at higher doses of NPs. In our study, enzymatic activity
347 changes observed in bulk soil and the rhizosphere treated with NPs (in the presence of a low dose of
348 CeO₂-NPs) did not correlate with a modification in bacterial community structure or size. Rather,
349 these changes were linked to an inhibition of their enzymatic activities (potentially in aerobic
350 bacteria), reduction of bacterial activity (that is undetected using a DNA-based approach), or to
351 changes in microbial communities other than bacteria.

352

353 *Canola potentiates the nanoceria impact on microbial activity and community structure*

354 Measuring the activity of several enzymes in soil is a suitable method to estimate the overall microbial
355 activity and its response to prevalent pollution.³⁸ Our results indicate that catalase, β-glucosidase and
356 alkaline phosphatase activities were inhibited by pristine nanoceria at 1 mg.kg⁻¹. These activities were
357 gradually impacted in terms of magnitude and type of the enzymes affected, progressing from the bulk
358 soil to the rhizosphere.

359 Similarly, we found a significant impact of pristine CeO₂-U on the selection of the microbiota in the
360 root compartment, based on the analysis of UniFrac distance metrics, phyla, and OTUs. Nanoceria did
361 not significantly affect the microbiota of bulk soil or the rhizosphere, in comparison to controls. Thus,
362 based on soil enzyme activities and microbial community composition, we have uncovered a

363 ‘nanoceria impact gradient’ progressing from bulk soil to the root-soil interface, indicating that the
364 plant potentiates the impact of these NPs in soil.

365 Terminal restriction fragment length polymorphism (T-RFLP) and PhyloChip analyses were recently
366 used to determine that nano-CeO₂-NPs (100 mg.kg⁻¹) do not affect soil bacterial communities in
367 unplanted soils, although they do trigger shifts in soybean rhizosphere communities.¹¹ No OTUs were
368 significantly correlated with nano-CeO₂ treatment, and the authors interpreted these results in terms of
369 an indirect effect of nano-CeO₂ on plant growth. Although the study did not quantify or analyze plant
370 root exudates, the authors suggested that the exudation could be reduced, due to the stunted growth of
371 plants exposed to 100 mg.kg⁻¹ of nano-CeO₂) or modified in composition, due to an abiotic stress of
372 CeO₂-NPs on plant roots. However, in our conditions, canola growth was not reduced based on the
373 above and belowground biomasses. We therefore conclude that the effect of CeO₂-NPs on plant root
374 microbiota is not only mediated by an effect on the plant, but that it could also originate from a direct
375 effect on bacteria. Pelletier and al. showed a size-dependent effect on the growth inhibition of
376 *Escherichia coli* by nanoceria.³⁹

377

378 ***Nanoceria generate microbiota with double-edged potential significance to plant and environmental***
379 ***health***

380 Our use of quantitative PCR and alpha diversity estimators indicates that the three nanoceria examined
381 in this study did not alter biomass or bacterial diversity, as compared to the controls. However, pristine
382 large-size CeO₂-U NPs significantly affected the community structure (in comparison to controls), as
383 seen in the analysis of UniFrac distance metrics. CeO₂-U treatment resulted in an increase in
384 *Burkholderiales*, in the *Comamonadaceae* family, especially in *Acidovorax* (5.65%), *Pelomonas*, and
385 *Methylibium* genera. (Table S6). In the root compartment, the total abundance of these OTUs reached
386 68.06% in the abundant community members for CeO₂-U treatment vs 9.51% in the controls (Table
387 S6). Alternatively to Ribosomal Database Project (RDP) classifier pipeline, OTU sequences can be
388 resolved using NCBI’s Taxonomy. Based on BLAST taxonomic assignment, the closest relatives to
389 these OTUs were *Acidovorax radialis* N35, *Pelomonas saccharophila*, *Methylibium petroleiphilum*
390 *PM1* and *Methylibium fulvum* (reclassified as *Rhizobacter fulvum*).⁴⁷ All these bacteria are reported as

391 degraders of polycyclic aromatic hydrocarbons (PAHs) in soil.⁴⁸⁻⁵⁵ Triggering the rise of microbial
392 genera that potentially degrade PAHs can be advantageous for remediation of hydrocarbon in soils.
393 However, hydrocarbon-degrading properties in bacteria can come with heavy metal tolerance, as well
394 as resistance to antibiotics and multidrug resistance⁴⁰⁻⁴², potentially in *Methylibium* and *Acidovorax*
395 genera.^{43,44} A functional gene analysis is needed to demonstrate this hypothetical trend. Yergeau et al.
396 identified *Methylibium* in a microbial community active in the rhizosphere of willow planted in HAP-
397 contaminated soils.⁴⁵ They show that genes related to hydrocarbon degradation together with antibiotic
398 resistance were more expressed in the HAP-contaminated rhizosphere. This underscores the need for
399 caution when disseminating ENMs that could exert a selection pressure conducive of microbial
400 blooms potentially resistant to heavy metals and antibiotics.

401 CeO₂-U NPs significantly decreased the abundance of OTUs in *Actinobacteria* (including the
402 *Actinosynnemataceae* and *Micrococcaceae* families and *Streptomyces*), *Gammaproteobacteria* and
403 *Betaproteobacteria* within the *Burkholderiales* (*Janthinobacterium*), and *Xanthomonadales*
404 (*Pseudoxanthomonas*) families (Table S6). These taxa are have been identified as the most dynamic
405 taxa associated with disease suppression in soil.⁴⁶ Among the ACM, *Janthinobacterium*
406 (*Oxalobacteraceae*) was enriched in the root compartment of control plants (12.4%), but inhibited in
407 the CeO₂-U treatments (3.0%). A significant decrease in the *Streptomyces* and *Pseudoxanthomonas*
408 genera was observed in the root compartment of the CeO₂-U treatment (0.7% and 0.6%, respectively),
409 in comparison to the control (6.1 and 4.6%, respectively). *Janthinobacterium*, *Streptomyces* and
410 *Xanthomonadaceae* are identified in consortia associated with soil suppressiveness against plant
411 pathogens.⁴⁷⁻⁵⁰ Importantly, disease suppressiveness of soil can help to reduce pesticide use.
412 *Janthinobacterium* and *Xanthomonadaceae* are both dominant taxa that exhibit a positive association
413 with plant shoot weights in wheat,⁵¹ peach tree⁵⁰ and rice,⁵² suggesting a beneficial role for these
414 bacterial groups. Thus, CeO₂-U treatment mostly affected families or genera that are beneficial to
415 plant health and growth.

416

417 ***Nanoceria design determines their impacts on microbial enzyme activities and bacterial community***
418 ***structure***

419 In this study, we aimed to examine how particle size or coating could modulate the impact of NPs on
420 microbiota activity and composition in bulk soil, rhizosphere, and root compartments.

421 This work revealed a gradient effect for the impact on microbial activity and community structure,
422 according to the design of nanoceria. Constrained PERMANOVA of the unweighted UniFrac distance
423 showed significant differences in coated CeO₂-NPs vs CeO₂-R, and in CeO₂-R vs CeO₂-U, in the
424 rhizosphere (Table S4). These results demonstrate that particle size and coating trigger differences in
425 the microbiomes. However, even if some phyla or OTUs were able to significantly discriminate
426 between these treatments (P-value <0.05), the Q-values surpassed the FDR 0.25 threshold (data not
427 shown).

428 Pristine aggregates of CeO₂-U NPs significantly altered the microbiota composition in the root
429 compartment in comparison to controls, CeO₂-R and coated CeO₂, whereas pristine small CeO₂-R NPs
430 did not show any impact (in comparison to the controls). Thus, particle size appears to be a key
431 parameter in modulating the fate of CeO₂-NPs in the rhizosphere, as well as access to the soil-root
432 interface. This conclusion needs to be confirmed with other NPs before being extended.

433 The mobility of CeO₂-NPs in soil depends on the soil organic matter content, and on the surface
434 coating of the NPs. Recent work has shown that alginate-coated NPs have higher mobility than
435 uncoated NPs, in sandy loam soil with very low organic matter content.⁵³ However, surface coating
436 decreases the NP mobility in soil solution, when the soil is enriched in organic matter. Exudation of
437 small organic molecules increases the concentration of organic ligands in the vicinity of the root
438 system. The competition of these organic molecules for adsorption onto the cerium surface is certainly
439 a factor that will increase the mobility of uncoated nanomaterials.

440 Water moves through the soil to the plant root and then to the transpiring leaves along pressure
441 gradients: these comprise suction (negative pressure) gradients in the soil, and diffusion pressure
442 deficit gradients in the plant.⁵⁴ It can therefore be assumed that NP diffusion in soil originates from
443 these pressure gradients. One recent study of porosity and hydraulic conductivity of the rhizosphere
444 has shown that bulk soil is better at retaining water than the rhizosphere.⁵⁵ Soils influenced by roots
445 (rhizosphere soil) are less porous due to increased aggregation, in comparison to bulk soil. Based on
446 size exclusion, it is likely that large-size and aggregated CeO₂-U NPs can diffuse more easily at the

447 root interface, whereas smaller size NPs could be hindered in their interaction with sites inside the
448 microporosity zone.⁵⁶

449 As stated above, pristine aggregates of CeO₂-U enabled the selection of bacterial genera those
450 members are identified as PAH degraders. Bacteria that degrade PAHs utilize an efficient oxidase to
451 promote the first step of hydrocarbon conversion, producing reactive oxygen species. Potent
452 mechanisms for reducing oxidative stress are thus required in all aerobic microorganisms that produce
453 oxygenase-type enzymes to metabolize pollutants.⁵⁷ CeO₂-NPs (IV) display a catalase mimetic activity
454 that is resistant to phosphate anions, pH changes and composition of cell culture media.²³ As indicated
455 by XANES,²⁰⁻²² the nanoceria used in this study have a (IV) oxidation state. CeO₂-R and coated CeO₂
456 exhibited catalase mimetic activity *in vitro*, which are likely to be retained in soil. Among the
457 nanoceria that we examined, only CeO₂-U, which lacks catalase activity, promoted the selection of
458 genera comprising hydrocarbon degraders potentially endowed with resistance to oxidative stress.
459 Many previous studies have shown that the catalase activity of nano-CeO₂ eases the impact on
460 organisms.⁵⁸⁻⁶¹

461 Pristine small-size CeO₂-R and aggregates of CeO₂-U, which display comparable surface charges, had
462 similar effects on the hydrolytic enzymes tested. The two pristine NPs both decreased β-glucosidase
463 and alkaline phosphatase enzyme activities, suggesting comparable interactions between the two types
464 of pristine NPs and the exocellular enzymes investigated (independent of particle size and initial
465 aggregation state). Conversely, citrate-coated CeO₂ particles did not affect these activities.
466 Compositional differences in microbial communities were not responsible for the observed functional
467 differences of CeO₂-NPs in the rhizosphere. Charge interactions could thus explain the inhibition of
468 these hydrolytic exocellular enzymes. Indeed, CeO₂-NPs must be in direct contact with cells in order
469 to display toxicity, *via* the reduction of Ce(IV) to Ce(III).^{22, 62} Citrate coating tends to decrease the
470 interaction of NPs with cells, as well as the Ce reduction kinetics.⁶³ However, positively charged
471 CeO₂-NPs become negatively charged in soil, due to the adsorption of phosphate ions or citrate (and
472 potentially carbonate ions, in the case of an alkaline soil).⁶⁴ These new negatively charged sites at the
473 surface can promote hetero-aggregation of CeO₂-NPs with natural colloids, such as clays or metallic
474 oxides. Citrate-stabilized and bare Ag-NPs showed similar sorption to silt particles in low-carbon soil,

475 suggesting that the surface charge does not control Ag-NP sorption to silt.⁶⁵ Nevertheless, the citrate
476 ligand is capable of forming a chelate through more than one coordinating group. Therefore, this
477 coating could act as a cross-linker to associate CeO₂-NPs with colloids in a different way than the
478 negative surface charges promoted by phosphate or carbonate ions.

479 Citrate-coated NPs tend to have a low effect on enzyme activities and on the bacterial community
480 structure. However, this lack of an impact may be misleading in the vicinity of the root, due to the
481 high variability in most of the parameters measured in the rhizosphere and the root compartment
482 (biomass in the rhizosphere, UniFrac distance metrics and relative abundance of phyla in the root
483 compartment) for citrate-coated CeO₂-NP treatment (Table S3, Fig. 1, Fig. S4), which precluded
484 significant differences with other treatments. Plant roots release small organic acids that can
485 remobilize organic compounds, such as HAPs by desorption from soil aggregates.⁶⁶ Citrate coating of
486 NPs can readily exchanges with dissolved organic matter (DOM) in soil.⁶⁷ We hypothesize that a
487 progressive and random release/exchange of the coating with DOM near the root, allows NPs to
488 interact with different colloid fractions, ions or cells, which triggers a variable behavior in the
489 rhizosphere and root compartment. We hypothesize that a progressive and random release/exchange of
490 the coating, near the root, allows NPs to interact with different colloid fractions, ions or cells, which
491 triggers a variable behavior in the rhizosphere and root compartment.

492 Altogether, our results show that nanoceria reduce microbial enzymatic activities and alter the
493 bacterial community structure, and that these effects spatially increase along a gradient progressing
494 from bulk soil to the root compartment. The design of nanoceria, including particle aggregation and
495 coating, clearly determines the amplitude of this impact. The primary effect occurred for aggregated
496 pristine nanoceria, which were deprived of catalase activity. Contamination of soil with pristine
497 aggregates of nanoceria conferred a selective advantage to genera whose common members are
498 hydrocarbon-degrading bacteria, which are potentially more resistant to oxidative stress, heavy metals
499 and antibiotics. On the other hand, several families and genera, whose members can be involved in
500 disease-suppressive activity toward plant pathogens, were inhibited in the root compartment of canola.
501 The finding that these effects occurred at the lowest dose ever tested in soil (1 mg.kg⁻¹) is of particular
502 concern, and invites further research in order to identify the resilience of the ecosystem. Nevertheless,

503 when 4-nm sized CeO₂-NPs were tested, the impact of nanoceria was limited to enzymatic activities.
504 This shows that nanoceria can be designed to ease the impact on soil microorganisms.

505

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512

513 **Supporting Information**

514 Supporting Information includes detailed protocols of enzymatic activity experiments, sequencing and
515 post-run analyses, catalase mimetic activity of nanoceria, microbial diversity (PCoA plots and
516 composition at the phylum level), plant biomass, physicochemical properties of the nanoceria and oil
517 used in these experiments, and bacterial biomass. This material is available free of charge *via* the
518 internet at <http://pubs.acs.org>.

519

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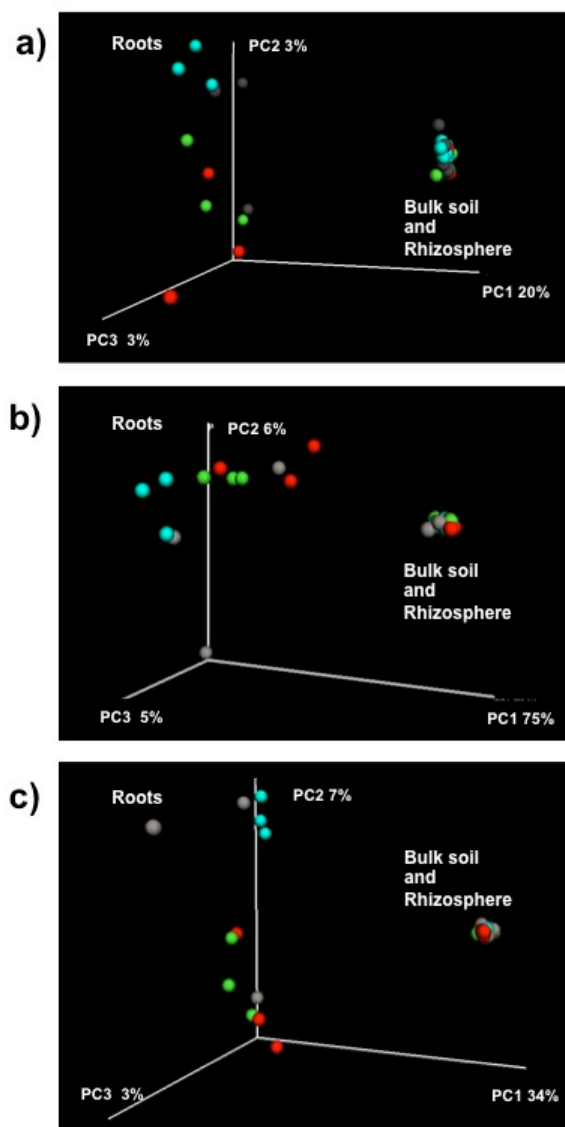


Figure 1: 3D beta diversity presented as PCoA plots depicting the clusters of bacterial communities within the different compartments (left: roots; right: rhizosphere and bulk soil) for the nanoceria treatments CeO₂-R (green), CeO₂-U (blue), coated CeO₂ (grey) and controls (red). Unweighted UniFrac (a), weighted UniFrac (b), and Bray Curtis (c) distance metrics. The percent variation explained by the PCs is indicated on the axes and refers to the fraction of the total variance. Statistical analysis (PERMANOVA) of unweighted and weighted UniFrac as well as Bray-Curtis distance metrics revealed clustering of microbial communities based on the soil compartment factor (Fig. 1a, $P=0.0001$; Fig. 1b $P=0.0001$; Fig. 1c, $P=0.001$) but not on nanoceria treatment ($P>0.05$).

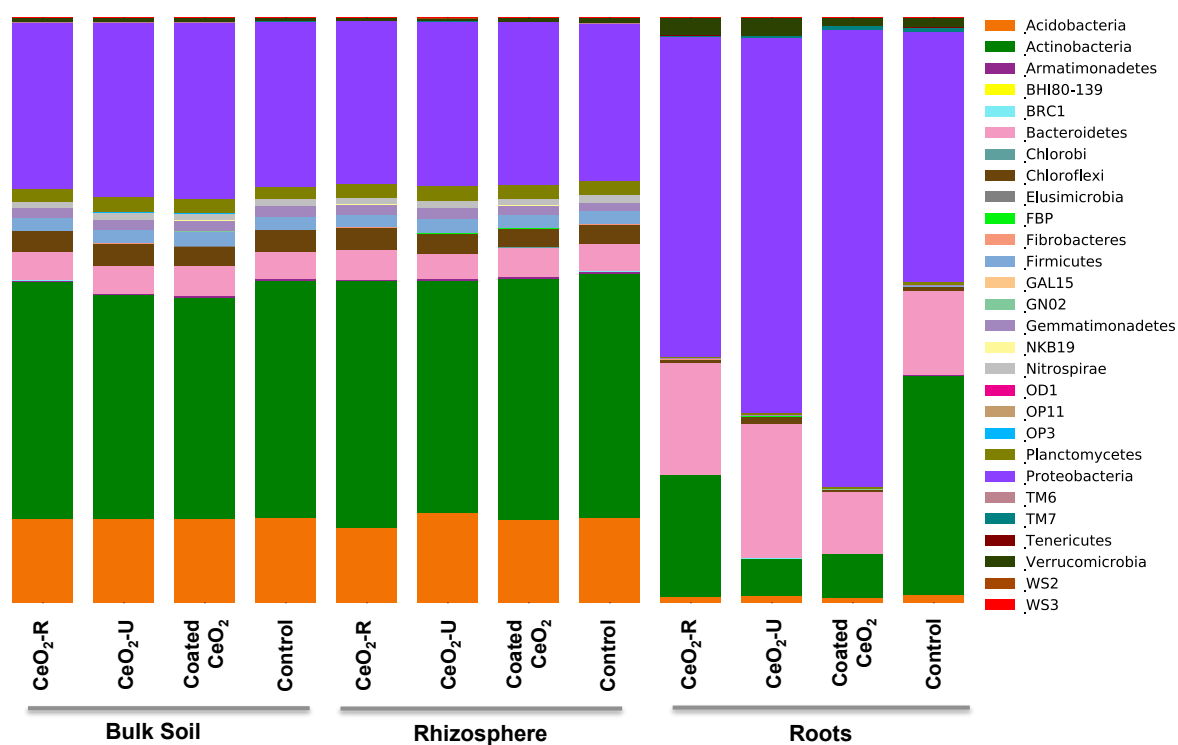


Figure 2: Relative abundance of major phyla in bulk soil, rhizosphere and root compartments for soil enhanced with nanoceria (pristine $\text{CeO}_2\text{-R}$, $\text{CeO}_2\text{-U}$ and citrate-coated CeO_2) at 1 mg.kg^{-1} and the respective controls. Significant differences were found in the root compartment and are summarized in Table S5.

Table 1: Enzymatic activities in the rhizosphere of canola and in bulk soil after 30 days of exposure to nanoceria. Samples include pristine CeO₂-R and CeO₂-U, and coated CeO₂ (1 mg.kg⁻¹) and controls. Values in bold denote statistically significant differences from controls (one-way ANOVA, post-hoc Student-Newman-Keuls test; P<0.05)

Enzymatic activity	Rhizosphere				Bulk Soil			
	Control	CeO ₂ -R	Coated-CeO ₂	CeO ₂ -U	Control	CeO ₂ -R	Coated-CeO ₂	CeO ₂ -U
Catalase μmol KMnO ₄ g ⁻¹ dry soil min ⁻¹	5.4 ± 0.4	4.3 ± 0.6	4.2 ± 0.2	4.4 ± 0.1	3.9 ± 0.3	3.7 ± 0.2	3.5 ± 0.3	3.2 ± 0.1
β-Glucosidase μmol p-NP g ⁻¹ dry soil h ⁻¹	1.28 ± 0.01	1.037 ± 0.07	1.16 ± 0.08	1.05 ± 0.11	0.93 ± 0.07	0.86 ± 0.03	1.06 ± 0.01	0.90 ± 0.05
Peroxidase μmol h ⁻¹ g ⁻¹	0.170 ± 0.002	0.154 ± 0.014	0.152 ± 0.006	0.168 ± 0.015	0.158 ± 0.030	0.129 ± 0.023	0.157 ± 0.033	0.145 ± 0.003
Phenoloxidase μmol h ⁻¹ g ⁻¹	0.221 ± 0.008	0.213 ± 0.03	0.210 ± 0.004	0.214 ± 0.014	0.238 ± 0.029	0.189 ± 0.058	0.194 ± 0.038	0.173 ± 0.004
Acid Phosphatase μmol p-NP g ⁻¹ dry soil. h ⁻¹	0.78 ± 0.03	0.71 ± 0.06	0.72 ± 0.08	0.69 ± 0.11	0.63 ± 0.04	0.60 ± 0.08	0.72 ± 0.15	0.59 ± 0.01
Alkaline Phosphatase μmol p-NP g ⁻¹ dry soil. h ⁻¹	0.90 ± 0.04	0.73 ± 0.10	0.79 ± 0.05	0.73 ± 0.04	0.63 ± 0.03	0.61 ± 0.02	0.65 ± 0.03	0.61 ± 0.07
Protease μg l-tyrosine released . g ⁻¹ dry soil h ⁻¹	133 ± 33	105 ± 16	119 ± 18	123 ± 17	93 ± 20	110 ± 16	104 ± 18	90 ± 28

p-NP: para-nitrophenyl

TOC Abstract :

