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1 **Assessing implications of nanoplastics exposure to plants with** 2 **advanced nanometrology techniques**

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11

12 **Abstract**

13 Despite the increasing attention given to the impacts of nanoplastics in terrestrial environments, there
14 is limited data about the effects on plants, and the quantitative information on uptake. In the present
15 study, wheat plants grown in hydroponics were exposed to Pd-doped nanoplastics. This allowed us to
16 quantify nanoplastics uptake and translocation to the shoots. Visualization of nanoplastics in roots was
17 performed with synchrotron micro X-ray fluorescence (μ XRF). Nanoplastics accumulated on the root
18 epidermis, especially at the root tip and in root maturation zones. A close relationship between plant
19 roots, rhizodeposits and nanoplastics behaviour was shown. Reinforcement of the cell wall in roots was
20 evidenced using Fourier transform infrared spectroscopy (FTIR) and synchrotron-computed
21 microtomography (μ CT). Synchrotron-computed nanotomography (nanoCT) evidenced the presence of
22 globular structures but they could not be identified as nanoplastics since they were observed both in
23 the control and treated roots. By utilizing the inorganic tracer in the doped-nanoplastics, this study
24 paves the road for elucidating interactions in more complex systems by using an integrative approach
25 combining classical phytotoxicity markers with advanced nanometrology techniques.

26 **Keywords:** quantification, microscopy, cell wall, root anatomy.

27 **Abbreviations:** X-ray fluorescence (μ XRF), Fourier transform infrared spectroscopy (FTIR),
28 synchrotron-computed microtomography (μ CT), yncrotron-computed nanotomography
29 (nanoCT), scanning electron microscopy (SEM), inductively coupled plasma mass spectrometry
30 (ICP-MS), pyrolysis gas chromatography–mass spectrometry (Py-GC/MS), polystyrene (PS),
31 polyacrylonitrile (PAN), dynamic light scattering (DLS), thermogravimetric analysis (TGA), total
32 organic carbon (TOC), low weight organic acids (LWOAs), instrument limit of detection (LOD),
33 contrast transfer function approach (CTF), linear discriminant analysis (LDA), principal
34 component analysis (PCA), least absolute shrinkage selection operator (LASSO), area under
35 curve (AUC) receiver operating characteristic (ROC), Inorganic engineered nanoparticles (ENPs).

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1. Introduction

41 As industrialization and population growth continue to place increasing pressures on the natural
42 environment, there has been an increased awareness that careful (re)use of our resources are
43 necessary to secure adequate food and water supplies, as highlighted by e.g. the U.N.
44 Sustainable Development Goals(Kanter et al., 2018). Soil nutrient quality and function are
45 fundamental for productive agricultural systems, yet several current strategies to promote
46 nutrient cycling or decrease water usage inadvertently introduce materials into the soil, such as
47 nano- and microplastics. This is notable the case of the reuse of processed sewage sludge as
48 agricultural fertilizer(Kelessidis and Stasinakis, 2012) and the use of plastics in plastic mulch
49 films, greenhouse materials and soil conditioners (Ng et al., 2018; Steinmetz et al., 2016).
50 Atmospheric deposition onto land also contributes to plastic contamination (Bergmann et al.,
51 2019). Data on particulate plastic concentrations in soils is still scarce and differ by several orders
52 of magnitude for microplastics (from a few to a few thousands of $\text{mg}\cdot\text{kg}^{-1}$ and/or $\text{particles}\cdot\text{kg}^{-1}$
53 depending on the studies) (Corradini et al., 2019; Scheurer and Bigalke, 2018; Wu et al., 2021;
54 Zhang and Liu, 2018). The occurrence of nanoplastics in a natural soil has only recently been
55 proved (Wahl et al., 2021) and information about their concentrations is not yet available. The
56 synthetic nature and persistence of particulate plastics may qualify these particles to be drivers
57 of environmental change, but holistically the impacts to both soil quality and plant growth and
58 productivity have only recently begun to be investigated (Chae and An, 2018; Horton et al., 2017;
59 Rillig et al., 2019; Rillig and Lehmann, 2020; Wu et al., 2021). Literature regarding the interaction
60 of plants with plastic debris is still scarce, but there is an increasing number of studies being
61 focussed on the effects caused by nanoplastics in plants.

62 A recent article demonstrated that microplastics can enter the root of wheat and lettuce(Li et
63 al., 2020). Nanoplastics particles may have a greater potential to cross plant barriers. Indeed,
64 studies have shown that a large number of nanoplastics can enter roots(Jiang et al., 2019; Sun
65 et al., 2020) and be transported to the aerial parts of plants(Chae and An, 2020; Li et al., 2020;
66 Lian et al., 2020; Liu et al., 2022; Zhang et al., 2019a). However, uptake and transport
67 mechanisms are still unclear and quantitative information is really limited, which is key to assess
68 environmental impact and potential risk to human health. Root tips have been identified as one
69 of the main points of entry(Jiang et al., 2019; Lian et al., 2020; Liu et al., 2022; Zhang et al.,
70 2019a), root hairs and the epidermis of maturation zone of roots have also been proposed (Sun
71 et al., 2020). Once inside the plant, apoplastic pathway seems to be the main transport
72 mechanism (Li et al., 2020; Liu et al., 2022; Sun et al., 2020), although the symplastic pathway
73 cannot be excluded with the available data. To date, only one study has quantified nanoplastics
74 in plant tissues. The authors used a four step extraction procedure followed by pyrolysis gas
75 chromatography–mass spectrometry (Py-GC/MS) to quantify PS and PMMA nanoparticles in
76 cucumber plants after hydroponic exposure(Li et al., 2021). The variety of individual plant
77 responses observed in previous studies in terms of growth, chlorophyll contents and/or
78 oxidative stress (Larue et al., 2021; Wu et al., 2021; Yin et al., 2021) points out the need to
79 develop new experimental approaches to allow for the tracing of this analytically challenging
80 target material and to provide a basis of interpretation of the reported effects.

81 This study aimed at evaluating the uptake and impacts of nanoplastics on wheat (*Triticum*
82 *aestivum L.*), a model plant and staple food source, and at providing a proof of concept of a new
83 combination of approaches including classical phytotoxicity markers and advanced imaging and
84 nanometrology techniques. We have used polystyrene (PS) nanoparticles doped with Pd. [In
85 order to specifically study the effects of particle morphology on plant response, two different](#)

86 nanoplastics morphologies have been used, so called smooth (S) and raspberry (R). These
87 nanoplastics present a core-shell structure with a core of Polyacrylonitrile (PAN) containing the
88 metal tracer and a shell of cross-linked PS (Mitrano et al., 2019). The stability of these
89 nanoplastics and their utility to study the fate of nanoplastics in complex organic matrix such as
90 sewage sludge or animal models has been demonstrated in previous studies (Clark et al., 2022;
91 Frehland et al., 2020; Keller et al., 2020; Lahive et al., 2022; Redondo-Hasselerharm et al., 2021).
92 Polystyrene (PS) is one of the most commonly produced commodity seven polymers(Geyer et
93 al., 2017). Its detection in organic fertilizers and compost(Brinton, 2005; Weithmann et al., 2018)
94 as well as in soils (Chen et al., 2020; Fakour et al., 2021; Fuller and Gautam, 2016; Piehl et al.,
95 2018; Scheurer and Bigalke, 2018) suggests that it may be broadly present in agricultural soils.

96 The use of metal-doped nanoplastics expanded our analytical possibilities to techniques
97 normally reserved for metal analysis, such as synchrotron micro X-ray Fluorescence (μ XRF) and
98 inductively coupled plasma mass spectrometry (ICP-MS). μ XRF has been demonstrated to be a
99 unique technique to study the interaction of plants with engineering nanomaterials (ENMs), it
100 provides multi-elemental detection with lateral resolution providing important information to
101 understand uptake, transport and storage mechanisms(Castillo-Michel et al., 2017). Combining
102 this with Fourier transformed infrared spectroscopy (FTIR), micro and nano computed X-ray
103 tomography (μ CT and nanoCT) and scanning electron microscopy (SEM), we could additionally
104 assess effects on root architecture and particle-root interaction. FTIR provides insights on the
105 cell wall composition and possible shifts of some families of molecules (Canteri et al., 2019), with
106 the appropriate chemometric analysis, it is an ideal technique for the screening of plant
107 response against stress when previous knowledge is scarce and/or contradictory, as it is the case
108 for micro and nano plastic exposed plants. X-ray computed tomography allows for the 3D
109 reconstruction of scanned objects, it is non-destructive and requires minimal sample
110 preparation avoiding the production of surface artefacts. It has been extensively used in the
111 study of plant root traits (Dhondt, 2012; Gao et al., 2019). In the present study, we used μ CT to
112 assess possible impacts of the nanoplastics on root structure and nanoCT to search for
113 nanoplastics in the root tissues. Although FTIR has been successfully applied to investigate the
114 interaction of nanomaterials with different plant tissues (Bakshi et al., 2019; Liné et al., 2021;
115 Savassa et al., 2021) and lab bench μ CT has been used for the visualization of ENMs in plant
116 roots (Avellan et al., 2017; Pradas del Real et al., 2017b), to our knowledge, this is the first time
117 that FTIR and synchrotron μ and nanoCT are used together to study the effects of nanoplastics
118 on plant root structure. Besides, thanks to the high flux and coherence of synchrotron source,
119 synchrotron based CT provides images with higher quality than lab-bench CT and allows the
120 detection of phase contrast which is about 2 orders of magnitude more sensitive than
121 absorption contrast (obtained in lab CT scanners). This information is of high relevance since
122 plant roots are the first barrier of the plant to be in contact with nanoplastics, they regulate
123 nutrient and water uptake by plants and they are drivers of many ecological functions (Freschet
124 et al., 2021). Using an integrative approach, we not only assess classical phytotoxicity markers
125 for plant growth and quality, but developed analytical tools to assess effects on cell walls (with
126 FTIR-ATR, μ CT, nanoCT and SEM) as well as use metal-doped nanoplastic particles to more
127 clearly assess interactions, uptake and transport (with μ XRF and ICP-MS). In particular, we aimed
128 to 1) quantify the uptake on nanoplastic and localize the main transport and accumulation in
129 tissues, 2) study the influence on physiological development (biomacromolecule composition,
130 root morphology and root exudation), and 3) provide further development of powerful
131 analytical tools which can help to facilitate more detailed and careful research into the
132 interaction of nanoplastics with plants in the future.

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2. Methods

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2.1. Pd-doped nanoplastics

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Emulsion polymerization of inorganic metal-doped nanoplastics spheres were made in house (Mitrano et al., 2019). Briefly, the procedure consisted of a two-step process in which first the particle core was synthesized (which contained the metal), after which a further shell of polystyrene was grown through feeding a second monomer-containing solution over time to augment the surface chemistry and morphology of the original particle. For the nanoplastics core, a surfactant (SDS) and initiator were charged inside the reactor. When the initial nucleation point was achieved, the dissolved metal precursor was added slowly to the reaction vessel. The evolution of the polymer conversion and particle size were followed through dynamic light scattering (DLS) (Malvern Zetasizer Nano Z, backscatter angle 173o at 25 °C) and thermogravimetric analysis (TGA) at T=120 oC (Mettler Toledo), respectively. After a conversion rate of approximately 90% was achieved, the shell feed was directly plumbed into the reaction vessel along with an additional shot of initiator and water. The shell feed, a mixture of water, styrene, DVB and SDS, was dosed for four hours. Two distinct shell morphologies were used in this work; so called smooth (S) and raspberry (R), with average diameters of approximately 160 nm (supplementary figure 1). Using these two different metal-doped nanoplastics allowed us to specifically isolate and study the potential impacts of particle morphology, since the other particle characteristics (e.g. agglomeration state, surface charge, chemical composition) were similar. The total Pd content was approximately 0.5% for both types. The concentration of nanoplastics in the stock solutions were between $6.22 \cdot 10^{16}$ to $8.88 \cdot 10^{16}$ particles \cdot mL $^{-1}$.

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2.2. Assessment of Pd stability in nanoplastics

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Stability of the Pd tracer inside the nanoplastics is paramount throughout the experiments as we rely on the Pd signal to assess nanoplastics uptake and localization. To assess the possibility of Pd leaching over time inside the plant, we simulated the conditions inside the plant cell environment by exposing the nanoplastics (corresponding to 1 mg Pd \cdot L $^{-1}$) to a nutrient solution (10 mL) containing KNO₃ (150 mM), NH₄H₂PO₄ (50 mM), MgSO₄ \cdot 7H₂O (0.5 mM), Ca(NO₃)₂ \cdot 4H₂O (100 mM and NaCl (5 mM), oxalic acid (5 mg \cdot L $^{-1}$), glucose (10 mg \cdot L $^{-1}$) and BSA (2.5 mg \cdot L $^{-1}$) initially adjusted to pH 5, in an end-over-end shaker for three weeks. To simulate ROS production in the plant, which had the potential to induce further Pd leaching, twice weekly spiked additions of H₂O₂ at low/realistic (7 mM) and high/extreme (70 mM) were made throughout the experiment. Nanoplastics suspensions, without the addition of salts or H₂O₂ in DI H₂O, were used as a control. Triplicate samples were sacrificed at weekly time intervals (i.e. 0, 1, 2, and 3 weeks) and passed through a centrifugal ultrafilter (10 kDa pore size) and filtrate was analyzed by ICP-MS to assess Pd leached from the nanoplastics over time. The quantification limit of Pd by ICP-MS was 10 ng \cdot L $^{-1}$.

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2.3. Plant culture

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Seeds of wheat plants were sterilized on 70% ethanol (v/v) for 5 min, rinsed with distilled water, and germinated in moisture vermiculite at 24°C under dark conditions. Once the cotyledon was developed (5 days), seedlings were transferred to a hydroponic system and allowed to acclimate for one week in 1/4 strength Hoagland nutrient solution: (0.75 mM KNO₃, 0.25 mM NH₄H₂PO₄, 0.13 mM MgSO₄ \cdot 7H₂O, 0.5 mM Ca(NO₃)₂ \cdot 4H₂O, 12.5 μM NaCl, 6.25 μM H₃BO₃, 0.5 μM ZnSO₄ \cdot 7H₂O, 0.5 μM MnSO₄ \cdot H₂O, 0.03 μM CuSO₄ \cdot 5H₂O, 0.13 μM (NH₄)₆Mo₇O₂₄ \cdot 4H₂O, 5 μM Fe(III)-EDTA), pH = 5.7. Wheat plants were exposed to the following treatments added to the nutrient solution: i) 3 mg \cdot L $^{-1}$ raspberry nanoplastics (3R) ; ii) 30 mg \cdot L $^{-1}$ raspberry nanoplastics (30R); iii) 3 mg \cdot L $^{-1}$ smooth nanoplastics (3S); iv) 30 mg \cdot L $^{-1}$ smooth nanoplastics (30S) and v) no

179 plastic addition (C). The nanoplastics suspension used to spike the nutrient solutions could
180 potentially contain some surfactants and residues from the synthesis process. In order to ensure
181 that observed effects were only due to the nanoplastics and not to any of these surfactants
182 and/or residues, we performed an additional control (CSN). To this end, the 30 mg·L⁻¹ raspberry
183 nanoplastics suspension was filtrated to remove the nanoplastics and the filtrate was used to
184 spike the nutrient solution. The hydroponic system consisted of 24 polypropylene pots
185 containing 0.7 L of the nutrient solution. Ten plants per pot were placed in floating polystyrene
186 (PS) disks of 15 cm diameter. The release of particles from these PS disks ($5.38 \cdot 10^8$ particles per
187 disk based on the determinations of Lambert et al. (2016) (2016)) was negligible compared to
188 the number of particles in the spiked solutions (between $5.22 \cdot 10^{15}$ and $7.45 \cdot 10^{17}$ in the 3 mg·L⁻¹
189 treatment and between $5.22 \cdot 10^{16}$ and $7.45 \cdot 10^{18}$ in the 30 mg·L⁻¹ treatment). Our choice of these
190 concentrations was mainly analytical – we aimed to have a plastic concentration where we
191 would be confident that we could measure the metal tracer above analytical detection limits.
192 However, it is worth noting that previous studies which have attempted to detect nanoplastics
193 in plants (not seedlings) used doses between 10 and 55 mg·L⁻¹ (Li et al., 2021; Lian et al., 2020;
194 Sun et al., 2020; Zhang et al. 2019a). Therefore, the dose of 30 mg·L⁻¹ used in the present study
195 falls within the range which has already been used in literature, and we were able to even
196 decrease this exposure concentration by 10 times (i.e. to 3 mg·L⁻¹), which further shows the
197 advantage of using metal-doped plastics, ICP-MS, and the microscopy techniques used here.

198 The system was continuously aerated and kept under controlled conditions (day/night
199 photoperiod 12/12h, 20/18°C, 70/60% humidity, 200/0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity).

200 Plants were randomly selected to be exposed to 3 or 30 mg nanoplastics L⁻¹, either smooth (S)
201 or raspberry (R). Nutrient solution without nanoplastics was used as a control. An additional
202 control was prepared to identify the potential effects of the solution in which the nanoplastics
203 were suspended, which may have contained trace concentrations of surfactant or
204 unpolymerized monomer from the synthesis process. The raspberry nanoplastics suspension
205 was centrifuged for 40 min at 60200 g to settle all nanoparticles into a pellet, then the
206 supernatant was decanted and added to the nutrient solution in the same volume as in the 30
207 mg L⁻¹ nanoplastics treatments. Four independent replicates were used for each treatment
208 (n=4).

209 A separate plant culture experiment was performed in similar conditions to prepare additional
210 samples for micro X-Ray fluorescence (μXRF) mapping. In this case, plants were grown in glass
211 pots containing 0.5 L of nutrient solutions.

212 **2.4. Analyses of the nutrient solution**

213 At each renewal of the nutrient solution, three parameters were measured in both the fresh
214 solution and in the solution after 3 or 4 days of contact with the plants (i.e. aged solution). The
215 hydrodynamic diameter and zeta potential of the nanoplastics were measured using a nano zeta
216 sizer (nanoZS, Malvern) for the 30 mg L⁻¹ dose only because the 3 mg L⁻¹ dose was below the
217 detection limit. The pH of the solutions was measured for all conditions at each time point. The
218 concentrations in total organic carbon (TOC) and low weight organic acids (LWOAs) were
219 determined on the last aged solution at the time of harvesting. TOC was measured with a TOC-
220 VCSN analyzer (Shimadzu). For LWOAs, an aliquot of 50 ml from each pot was frozen in liquid N₂
221 and freeze-dried. The freeze dried samples were re-suspended in 0.4 mM of heptafluorobutyric
222 acid and filtered at 0.20 μm . Then LWOAs were determined by ionic chromatography (Dionex
223 DX 500) using a conductivity detector. Chromatogram conditions are described in Pradas del
224 Real et al. (2017a).

225 **2.5. Harvesting and sample conditioning**

226 After three weeks of exposure, wheat plants were harvested, and their shoots and roots were
227 immediately separated at the root-shoot junction. Both roots and shoots were washed with
228 distilled water and weighed to determine fresh biomass. From each pot, different plant aliquots
229 were prepared for subsequent analyses. For the determination of physiological parameters,
230 subsamples were immediately frozen in liquid N₂ and stored at -80°C until analysis. For the
231 determination of dry mass, ICP-MS analyses and Fourier transform Infrared spectroscopy (FTIR),
232 subsamples were dried in an oven at 70 °C for 48 h and stored in a desiccator until analysis.

233 The roots tips and elongation zone of five roots per condition were prepared for analysis by
234 scanning electron microscopy (SEM) and synchrotron-based computed microtomography
235 (μCT). Samples were immediately fixed in 2.5% glutaraldehyde in 0.1M sodium phosphate buffer
236 (pH 7.2) and left overnight. Then they were washed (3 times, 10 min per wash) with 0.1 M
237 sodium phosphate buffer (pH 7.2) followed by serial transfer in ethanol series: 30%, 50%, 70%,
238 80%, 90%, 95%, 100% (2 times, 10 min duration). Samples were then subjected to critical point
239 drying (CPD) using a Leica EM CPD300 instrument.

240 For μXRF analyses, root samples were separated from shoots and thoroughly washed after
241 harvest. The apices were sectioned, frozen in isopentane, cooled in liquid N₂ and immediately
242 freeze dried at -50°C and 1·10⁻²mbar in an Emitech 750 freeze drier. Mature zone of the roots
243 were embedded in Optimal Cutting Temperature (OCT) resin (Leica), frozen in isopentane and
244 cross-sectioned (20μm) using a cryomicrotome (RM2265/LN22 Leica).

245 **2.6. Nanoplastics concentration in plant tissues**

246 Pd content in plant biomass was determined by induced coupled plasma mass spectrometry
247 (ICP-MS) after microwave assisted acid digestion. Approximately 100 mg of sample was placed
248 into a Teflon digestion tube, 1 ml H₂O₂ (30%, Sigma-Aldrich, USA) was added. After 1 h, 4 ml of
249 HNO₃ was added (in two separate aliquots of 2 ml each) and allowed to stand for an additional
250 hour. Finally, 0.5 ml of H₂SO₄ (Rotipuran® Supra 95 %, Carl Roth, Germany) was added. Samples
251 were subjected to 253°C at 100 bar over 25 min in a microwave digester (Ultraclave, MLS GmbH,
252 Germany). Pd concentrations were measured by ICP-MS (8900 ICP-MS Triple Quad, Agilent
253 Technologies, US) and Indium was used as internal standard. Calibration solutions ranging from
254 0.1 - 50 μg·L⁻¹ were produced from Pd standard solutions (Palladium standard for ICP 10,000
255 mg·L⁻¹, Sigma-Aldrich, US) in 2 % nitric acid (Rotipuran® Supra 69 %, Carl Roth, Germany). Pd
256 concentrations in samples were converted into nanoplastics (mass) concentrations based on
257 their average Pd content of 0.5%.

258 Based on calculated nanoplastics concentration in shoots and roots, the transfer factors (TF)
259 were determined as described in Supplementary Information.

260 **2.7. Nanoplastics distribution in roots**

261 After critical point drying, roots were coated with carbon and imaged by SEM with a FEG ZEISS
262 Gemini SEM 500, coupled with an energy dispersive X-ray analyzer (EDX, EDAX OCTANE ELITE
263 25) at 3 to 10 kV in high vacuum mode. The region examined was located at 400 μm from the
264 root tip. One to two roots per condition were analysed.

265 Freeze-dried root tips and fresh cross sections from the mature zone were studied by
266 synchrotron micro X-Ray fluorescence (μXRF) on the scanning X-ray microscope at ID21
267 beamline of the ESRF (The European Synchrotron Radiation Facility, Grenoble, France). Focusing
268 was done using a Kirkpatrick–Baez mirror system (0.7x0.5μm²). Fluorescence mapping was done

269 at 7.2 keVs for root apex and at 5.1 keVs for cross sections with dwell time of 100 ms. For root
270 cross sections, measurements were performed in cryogenic conditions using a vibration-free
271 cryo stage, passively cooled by a liquid nitrogen Dewar. In both cases, emitted fluorescence was
272 recorded on a silicon drift detector (SGX Sensortech 80 mm² active area) and drilled photodiode
273 was used as incoming intensity monitor. Elemental maps presented in this article were obtained
274 by fitting each pixel fluoresce spectrum using PyMCA (Solé et al., 2007) software as described in
275 Castillo-Michel et al. (2017). Images from the control processed by the fitting routine presented
276 a background signal in the Pd channels. In order to subtract this background from all control and
277 nanoplastics treated samples, filter masks were produced for epidermis and cortex/vascular
278 regions. From the average XRF spectrum of the control map the Pd fit area did not satisfy the
279 criteria of instrument limit of detection (LOD) defined as fit area \geq 3* standard deviation. For
280 treated maps the epidermis and cortex-vascular region masks were used to produce average
281 XRF spectra and the LOD criterion was satisfied only for the epidermis region. The maps in figure
282 1c present the Pd signal obtained from applying the filtering masks at the epidermis, the XRF
283 average spectra resulting from this analysis and the corresponding fitting areas and sigma values
284 are shown in supplementary figures 7 and 8.

285 The root samples prepared by critical point drying were mounted on top of quartz capillary to
286 be imaged using synchrotron-computed nanotomography at ID16B nano-analysis beamline at
287 the ESRF. Here a holotomography technique was used: four tomographies were acquired using
288 a beam energy of 29.5 keV and a flux of 1.5x10¹¹ ph/s. For each tomography, 3203 phase
289 contrast images with a pixel size of 25 nm were recorded over 360° using a PCO edge 5.5 camera.
290 The acquisition time per 2D projection was 30ms. The phase retrieval was performed using a
291 contrast transfer function approach (CTF) (Cloetens et al., 1999). The 3D volumes were
292 reconstructed via filtered back projection using the PyHST2 software at the ESRF (Mirone et al.,
293 2014). Segmentation was performed using Avizo (version 2019.3, Thermo Fischer Scientific Inc.,
294 Massachusetts) and Matlab (version 9.5.0 (R2018b), The MathWorks Inc., Massachusetts).

295 **2.8. Phytotoxicity markers**

296 The level of lipid peroxidation was determined in terms of malondialdehyde (MDA) content
297 according to Heath and Packer (1968) modified for these samples (Heath and Packer, 1968).
298 Photosynthetic pigments were analysed as described in Larue et al. (2014) (2014). The protein
299 content was determined according to Bradford method (1976) using bovine serum albumin
300 (BSA) as standard (Bradford, 1976). Further description of these method is provided in
301 Supplementary Information.

302 **2.9. Biochemical composition**

303 The effect of nanoplastics exposure on the biochemical composition of plant tissues was studied
304 by Fourier Transform Infrared Spectroscopy in Attenuated Total Reflectance mode (FTIR-ATR)
305 on freeze dried powdered samples. Samples were analysed using a FTIR spectrometer with an
306 ATR accessory (Thermo Nicolet NEXUS 470 ESR) over the range of 4000 – 400 cm⁻¹ with a spectral
307 resolution of 4 cm⁻¹. One spectrum was the average of 64 scans per sample. Four biological
308 replicates per treatment were collected, with four technical replicates performed for each
309 biological sample.

310 **2.10. Impact on root structure**

311 Roots prepared by critical point drying were mounted into small plastic vials and subjected to
312 synchrotron-computed microtomography in ID19 beamline at the ESRF. Scans were acquired at
313 26.5 keV, close to Pd K-edge (24.3 keV). In total 4000 projections were acquired with a pixel size
314 of 0.72 μ m and an acquisition time of 50 ms each over a rotation of 360°. Several scans along

315 the z axis were performed and merged in order to cover a sufficient field of view. Reconstruction
316 was done by exploiting the phase contrast using the Paganin's method applying a delta/beta
317 ratio of 300, coupled to conventional filtered back projection. The reconstructed 3D image made
318 of voxels corresponds to a map of the refractive indices stored in units of $2\pi/\lambda$, with λ being the
319 wavelength of the X-ray beam. This map is linearly related to mass density. The reconstructed
320 values are converted to 16bit integer values and referred to as gray values

321 For each sample, a region of interest was chosen within the elongation zone, such as to maximize
322 the analysed volume but neglecting regions with damaged tissue. The segmentation of the plant
323 cell walls and air, as well as the segmentation into different structural regions (epidermis, cortex
324 and stele) was achieved by an iterative approach combining Avizo (version 2019.3, Thermo
325 Fischer Scientific Inc., Massachusetts,) and Matlab (version 9.5.0 (R2018b), The MathWorks Inc.,
326 Massachusetts).

327 In a first step, a rough watershed algorithm was used to segment the cells from the surrounding
328 air. To separate cells that might have a connection after the rough watershed segmentation, a
329 'separate objects' step was performed on the 3D data. On a slice-wise basis, the cell area was
330 determined such that the structural regions (epidermis, cortex and stele) could be assigned
331 according to the cell size. By combining multiple morphological operations, masks for each
332 region were created. These masks were multiplied with the original respective gray value
333 volumes and a second watershed algorithm performed within a) the stele and b) within the
334 epidermis and cortex. These results were finally combined and cleaned with a 'separate objects'
335 step on the 3D data. This process resulted in masks for the segmented cell walls, the cells, and
336 the different regions of interest (i.e. epidermis, cortex and stele).

337 The areas and number of the segmented cells were analysed along the root length for the
338 epidermis, the cortex and the stele. For these cells, their volumes were also determined
339 individually for each of the three regions. The masks of the cell walls were used to determine
340 the local cell wall density in 3D. Based on averaged gray values along 20 μm from the root middle
341 region, three thresholds were established (high, medium and low) and used to produce masks
342 of cell wall density regimes. Subsequently, the number of pixels from each mask were extracted
343 to show the percent of pixels from each regime in each sample. Figure 16 in Supplementary
344 information shows the thresholding method. Since all samples were imaged and reconstructed
345 in the same way, the gray values (as a measure of the mass density) were compared between
346 the different tissue regions and samples. Figure 4 illustrates the different steps of this
347 segmentation process.

348 **2.11. Statistical analyses**

349 Statistical analyses were performed using the SPSS software. Normality and homogeneity of
350 variances were tested by Shapiro-Wilk and Levene's tests, respectively. The analysis of variance
351 for variables with homogeneous variances, was performed by one-way ANOVA followed by a
352 post-hoc Tukey's (HSD) test at the 5 % level. The variables with non-homogeneous variances
353 were analysed using Welch method followed by Dunnett post hoc test.

354 Pre-processed FTIR spectra were analysed using Orange Data Mining software (Demšar et al.,
355 2013). Linear Discriminant Analysis (LDA) together with Principal Component Analysis (PCA) was
356 applied to cluster the samples. Logistic Regression with Least Absolute Shrinkage as Selection
357 Operator (LASSO) was used to identify the main features contributing to differences between
358 treatments. The robustness of the model was monitored using a K-fold cross validation method.
359 The model's robustness parameters AUC (Area Under Curve), receiver operating characteristic

360 (ROC) curve, precision, and sensitivity (or recall) were reported. Boxplots of cell volumes were
361 performed using Pandas package in Python.

362

363

364 **3. Results and discussion**

365 **3.1. Nanoplastics are absorbed by roots and transported to the shoots**

366 Thanks to the use of Pd-doped nanoplastics, the present study provides quantitative information
367 about the uptake and transfer to the shoots of nanoplastics by a plant. In order to test the
368 stability of Pd in the nanoplastics, a leaching experiment was performed in DI H₂O and in
369 simulated plant cell environment with low or high H₂O₂ pulse additions over three weeks. While
370 a very small proportion of Pd was not incorporated into the nanoplastics upon initial synthesis
371 (0.5%), no further Pd release was measured over the duration of the experiment. In all tested
372 conditions, the concentration of free Pd was lower than 0.05% (Supplementary figure 2).
373 Therefore, Pd can be used as a proxy to calculate nanoplastics concentration in plant tissues but
374 the plant is not exposed to Pd itself, which is securely encapsulated within the plastic. Pd was
375 detected both in the plant roots and in shoots, meaning that nanoplastics were absorbed by
376 plant roots and translocated to the shoots (figure 1a). The total concentration of nanoplastics in
377 plant tissues was dose-dependent, however no significant differences were found between
378 different nanoplastics surface morphologies (smooth surface vs. rough surface so called
379 raspberry), where particle size and surface chemistry were otherwise similar (supplementary
380 figure 1 and 4). Transfer factors were significantly higher ($p < 0.05$) in 3 mg·L⁻¹ treatments than in
381 the 30 mg·L⁻¹. This may be related to the presence of larger agglomerates on the root surface in
382 the high dose treatment, which may limit the internalization and transfer of the individual
383 nanoplastics.

384 [Agglomerates of nanoplastics were observed at the surface of roots exposed to both 3 and 30](#)
385 [mg·L⁻¹nanoplastic \(figure 2 and Supplementary figure 3\). Nanoplastics were associated to a](#)
386 [network of filaments on the root surface.](#) These structures may belong to the root cap mucilage
387 (Tran et al., 2016) but they can also be produced by bacteria developed on the surface of the
388 roots (Gantar et al., 1995). [Bacteria were observed on the root surface in all conditions \(figure 2](#)
389 [and Supplementary figure 3\). They may correspond to the various species previously identified](#)
390 [in the rhizosphere of wheat \(Matos et al., 2005\), although no identification was performed in](#)
391 [this study.](#) In a recent study, Li et al. (2020) suggested that the entrapment of PS beads in the
392 root mucilage of wheat and lettuce promoted their adhesion on the root surface (Li et al., 2020).
393 Inorganic engineered nanoparticles (ENPs) differ in many aspects from nanoplastics. However,
394 at least those which are resistant to dissolution, such as TiO₂ and AuNPs, share some physico-
395 chemical properties and potential transformations (e.g., homo and hetero aggregation, changes
396 in surface charge, formation of an 'ecocorona') with nanoplastics (Syberg et al., 2015).
397 Therefore, some prior knowledge on ENPs behaviour in this system can be used to put our
398 results into perspective. The agglomeration of ENPs with root mucilage has been reported in a
399 wide variety of terrestrial plants and, in general, it has been related with a decrease in the uptake
400 and translocation to the shoots, with the exception of positively charged and/or small (i.e. < 20
401 nm) ENPs (Schwab et al., 2016).

402 [After 3 - 4 days of culture, an increase in the average particle hydrodynamic diameter was](#)
403 [observed for the nanoplastics of the 30S and 30R treatments that was accompanied by a](#)

404 decrease of their surface charge (Supplementary figure 4). These analysis of the nutrient
405 solution confirmed the agglomeration of nanoplastics during plant growth, due to the combined
406 effects of high ionic strength, root exudates and other rhizodeposits, and increased pH (more
407 details in Supplementary Information). Root exudates play a key role in plant nutrient
408 acquisition. Changes in the concentration of these compounds may provide some insights on
409 the impact of the presence of nanoplastics in the plant, as shown for plants exposed to metallic
410 and organic contaminants (Chaudhry et al., 2005; Dong et al., 2007). A recent study on a dicot
411 showed an increase in the exudation of oxalate in *Arabidopsis thaliana* exposed to PS
412 nanoplastics (2020). In the present study, the concentration of low weight organic acids (LWOAs)
413 was determined at the end of the culture. Different changes in concentration were found
414 depending on the of organic acids analysed (Supplementary figure 5). A significant increase
415 ($p < 0.05$) was found for the 3S treatment in the case of citric, malic, lactic and succinic acids and
416 for the 30S treatment in the case of oxalic acid. The concentrations of formic and acetic were
417 not affected by nanoplastics treatments. PCA-LDA performed on the organic acid concentration
418 data (supplementary figure 5b) show that their composition varied as a function of the type of
419 nanoplastics and dose, as shown by In addition, an increase in total organic carbon was found in
420 the nutrient solution of plants exposed to nanoplastics compared to controls (supplementary
421 figure 5a). These results are evidence of a complex response from the plant to nanoplastics,
422 affecting different classes of rhizodeposits.

423 The localization of the nanoplastics was studied by μ XRF, using the Pd signal, on entire root tips
424 (Figure 1b) and on root cryo-sections from the maturation zone (Figure 1b) of plants exposed
425 to $30 \text{ mg}\cdot\text{L}^{-1}$ nanoplastics. Nanoplastics were detected in the root cap and on the epidermis of
426 root maturation zone in association with the cell wall (Figure 1), but not in the cortex or in the
427 vascular cylinder (Supplementary figures 7 and 8). Previous studies have shown an accumulation
428 of nanoplastics at the surface of the root cap of *Vicia faba*, *T. aestivum*, *Arabidopsis thaliana* and
429 *Oryza sativa* with or without evidence of internalization (Jiang et al., 2019; Lian et al., 2020; Liu
430 et al., 2022; Sun et al., 2020; Taylor et al., 2020). The μ XRF results, combined with the evidence
431 of nanoplastics uptake and translocation, suggest that the root cap is a point of entry of
432 nanoplastics in wheat plants. A similar finding was presented for some ENPs into the plants. The
433 loose of junctions between cells in this location offer a pathway for small particles entry (Schwab
434 et al., 2016).

435 **3.2. Impacts on root architecture despite low phytotoxicity**

436 The exposure to nanoplastics did not induce negative effects on plant development in terms of
437 total biomass, oxidative stress or chlorophyll and protein contents (supplementary figures 9-12).
438 The absence of significant impacts on broad phytotoxicity parameters found in wheat plants is
439 in accordance with previous studies, showing no significant effects for *A. cepa* and *V. faba*
440 exposed to doses $< 100 \text{ mg}\cdot\text{L}^{-1}$ (Giorgetti et al., 2020; Jiang et al., 2019). Conversely, Lian et al.,
441 (2020) found positive but non-monotonic responses for *T. aestivum* (Lian et al., 2020). On the
442 other hand, Li et al. (2020) found different effects in *Cucumis sativus* exposed to $50 \text{ mg}\cdot\text{L}^{-1}$ of PS
443 nanoparticles depending on the size of nanoparticle and the parameter studied. Sun et al. (2020)
444 found different responses depending on the surface charge of the nanoplastics. Collectively, this
445 suggests that the ability of plants to modulate antioxidant levels and the expression of genes
446 related with oxidative stress in order to neutralize the effects of ROS production (Sun et al.,
447 2020) strongly depends on the specie investigated, the characteristics of the nanoplastics and
448 the dose. This also highlights the need to develop new approaches to measure other
449 phytotoxicity endpoints. As indicated in the previously mentioned studies, metabolic, cytologic

450 and genotoxic alterations may all be possible. Therefore, we developed a methodology to screen
451 for potential biochemical alterations caused in wheat tissues because of nanoplastics exposure
452 based on the use of ATR-FTIR together with a robust multivariate analysis.

453 PCA-LDA performed on ATR-FTIR spectra from roots and shoots showed clear clustering of
454 treatments (figure 3). The main FTIR bands explaining the differences among treatments were
455 selected by Logistic regression with a LASSO estimator are provided in the supplementary
456 material (Supplementary table 1). An increase in the absorbance of different bands related with
457 carbohydrates and cell wall components (cellulose, pectin and lignin) was found for 30 mg·L⁻¹
458 treatments and 3 mg·L⁻¹ smooth treatment in roots, which is indicative of a reinforcement of the
459 cell walls. Conversely, a decrease of these bands was observed in the shoots, suggesting weaker
460 cell walls. Additionally, in both roots and shoots, spectral variations were observed for IR bands
461 related to amides, which indicates that the protein pool was affected as well.

462 Synchrotron μ CT was used to further study the impacts of nanoplastics on root cell walls and
463 architecture (figure 4). Results confirmed the reinforcement of the cell walls in roots from the
464 30 mg·L⁻¹ smooth treatment. An increase in the proportion of voxels in the medium and high-
465 density regime was observed for this treatment in comparison to the control (Figure 5a and b).
466 This increase in cell wall density was mainly located in the epidermis of exposed roots, with some
467 significant spots located in the endodermis, likely corresponding to the Casparian strip (Fig 5 a
468 and b). Approximating the roots to cylinders (figure 4 a), the small differences in surface to
469 volume ratios among samples cannot account for the differences observed in the proportion of
470 density regions. A decrease in the volumes of epidermal cells was also observed in roots from
471 this treatment (Figure 5 c). 3D reconstructions of the analysed roots are provided in
472 Supplementary material. The 3D rendering of the gray values using a temperature color bar
473 clearly shows the higher proportion of voxels in the high-density regime in the surface of treated
474 roots, especially for the 30 mg·L⁻¹ smooth treatment. The adapted contrast images highlight that
475 this increase is found not only on the surface but also throughout the entire epidermal layer,
476 which is subsequently translated into a decreased cellular volume.

477 The reinforcement of the cell wall is a well-known response to biotic and abiotic stress
478 developed by plants to preserve cell wall integrity and cell turgor (Le Gall et al., 2015).
479 Furthermore, the adsorption of pollutants to the cell wall is widely described as a detoxification
480 mechanism in plants to reduce their presence in the cytoplasm, interference with cell
481 functioning (Krzyszowska, 2011), as well as to regulate transport through plant tissues (Song et
482 al., 2013). For example, some plant species exposed to divalent and trivalent metal ions increase
483 the biosynthesis of cell wall compounds to increase the trapping capability of the cell wall
484 (Krzyszowska, 2011). Fine μ XRF analysis showed the presence of nanoplastics within epidermal
485 cell wall (Figure 1c), so a similar mechanism is plausible in this study as well. However, the
486 reinforcement of root cell walls as a response to nanoplastics exposure seems to have a negative
487 impact to the cell walls of shoots, where a decrease in the absorption bands of the same
488 components can be indicative of the weakening of these structures. On the other hand, the
489 decrease in the cell volumes of the epidermis of roots exposed to 30 mg·L⁻¹ smooth nanoplastics
490 may be another consequence of the cell wall reinforcement as cell walls are responsible for the
491 mechanical control of cell size and shape (Le Gall et al., 2015). Collectively, these results suggest
492 that smooth nanoplastics have a more pronounced impact on cell wall composition and root
493 structure than raspberry nanoplastics. Although the raspberry nanoplastics had a bumpier
494 surface, the two types of nanoplastics had similar zeta potentials and sizes (Supplementary
495 Figure 4). So, with the available data, it was not possible to explain the difference found in the

496 [plant response against the two nanoplastics morphologies](#). Further investigations on other
497 physico-chemical parameters of the plastics properties which most influence uptake would be
498 necessary for further interpretation, which suggests that future studies should consider to use
499 an array of particle sizes, shapes and surface characteristics to understand the varied stress
500 responses of plants to plastics.

501 Alterations in the protein pool were also detected in both roots and shoots. Cross linked
502 structural proteins play an important role in the regulation of cell wall plasticity (Le Gall et al.,
503 2015), however, it is not possible to confirm if the observed changes are also related with the
504 cell wall conformation or with any of the other critical functions played by proteins in plant cells.
505 Alterations in carbohydrate and aminoacid metabolism have been recently reported in wheat
506 plants exposed to PS nanoplastics (Lian et al., 2020), so it is also plausible that the biochemical
507 changes observed here are the consequence of alterations in basic metabolic pathways. On the
508 other hand, some studies have shown a relationship between cell wall reinforcement and
509 changes in root exudation in plants under stress, (Feng et al., 2020) especially due to pathogen
510 infection (Brahmi et al., 2016; Castilleux et al., 2018; Vandana et al., 2019). If such a relationship
511 between the changes in biochemical composition of plant tissues and root exudation in wheat
512 exposed to nanoplastics exists remains to be elucidated. However, an increase in total organic
513 carbon in the nutrient solution due to the passive loss (senescence or turnover) of reinforced
514 cell walls in exposed plants cannot be excluded (de la Fuente Cantó et al., 2020).

515

516 **3.3. Advances in nanometrology to assess nanoplastics interactions with plants**

517 The carbonaceous nature of plastic makes its detection in organic matrices, such as plant tissues,
518 a challenge which is exacerbated in the case of nanoplastics due to their small size. This is one
519 of the reasons for the scarce number of studies regarding nanoplastics-plants interactions.
520 Recent studies using fluorescent-labelled PS nanoplastics have provided evidence of their
521 internalization by different plant species (Chae and An, 2020; Giorgetti et al., 2020; Jiang et al.,
522 2019; Li et al., 2020; Lian et al., 2020; Liu et al., 2022; Sun et al., 2020). However, the use of
523 fluorescent-labelled nanoplastics is limited to direct observation under a microscope and suffers
524 from artefacts such as fluorophore leaching and tissue autofluorescence. This approach also fails
525 to provide quantitative information about the uptake and distribution to different plant tissues.
526 The present study demonstrates that the use of Pd-doped nanoplastics is an excellent approach
527 to overcome these pitfalls as it allows the use of techniques such as synchrotron μ XRF and ICP-
528 MS. On one hand, the better detection limits of these techniques compared to that of
529 microscopic techniques previously used allowed us to detect nanoplastics in wheat plants at
530 lower and probably more realistic doses ($3 \text{ mg}\cdot\text{L}^{-1}$ vs. $10\text{-}100 \text{ mg}\cdot\text{L}^{-1}$). ICP-MS and XRF techniques
531 overcome the issue of matrix complexity by using a non-endogenous metal incorporation (Pd)
532 as the proxy for their fate in plant tissues. The detection of Pd by ICP-MS allowed us to quantify
533 the uptake of nanoplastics as a function of their concentration in the nutrient solution as well
534 as the relative transfer to the shoots, where they are found at much lower concentrations, which
535 has not been previously accessible by microscopic techniques. Taking further advantage of the
536 Pd labelling of the nanoplastics, the use of synchrotron μ XRF allowed us to identify the root cap
537 and the maturation zone of roots as the main points of accumulation and potential absorption
538 of nanoplastics in roots, while preserving the close to native environment thanks to cryogenic
539 conditions. It also allowed us to show the accumulation of nanoplastics within epidermal cell
540 walls. The spatial resolution of synchrotron XRF is given by the beam size, and current
541 technology allows one to focus the X-ray beam down to the nanoscale ($\sim 25\text{nm}$). In this work the

542 resolution was 1 μ m. Other points of entry described for ENPs, such as root hairs or cracks at
543 sites of lateral root emergences, may also be applicable for nanoplastics as well. For example, Li
544 et al. (2020) suggested that the latter could be a major point of entry for microplastics in wheat
545 and lettuce (Li et al., 2020). The broad resolution range of synchrotron XRF may allow to select
546 the adequate set-up to investigate these additional uptake mechanisms for Pd-doped
547 nanoplastics as well as their transport (symplastic vs apoplastic) in plant tissues. Quantification
548 of nanoplastics in plant tissues using Py-GC/MS is a promising technique recently tested and
549 shows good detection limit (Li et al., 2021). However, the advantage of using of Pd doped
550 nanoplastics over this approach is the possibility to have both quantitative and imaging
551 information over the same set of samples, which is an asset to study uptake and transport
552 mechanisms.

553 The innovative combination of ATR-FTIR with synchrotron μ CT has shown the primary role of
554 the cell walls in the response of wheat plants to nanoplastics exposure. Whether this is a
555 regulated response to control the damage produced by nanoplastics or a consequence of
556 metabolic impairments remains to be elucidated. Of note is the high correlation between
557 nanoplastics distribution shown by μ XRF and the increase in density observed in the epidermis
558 by μ CT. With the available data, it is not possible to conclude if nanoplastics accumulation has
559 contributed to increase gray value distribution (which is scaled with the mass density) in this
560 tissue region. However, taking into account the small size of nanoplastics regarding voxel size,
561 the potential contribution to phase contrast would be minimal in comparison to the one caused
562 by morphological alterations of the tissue. A control root and a root exposed to 30 mg-L⁻¹
563 smooth nanoplastics were investigated by synchrotron computed nanotomography (nanoCT).
564 Thanks to a pixel size of 25nm this technique provides a more detailed view of cellular structures
565 than μ CT and should allow the visualization of nanoplastics (160 nm). However, as it is shown in
566 figure 6 and supplementary figure 18, globular structures with sizes below 1 μ m that can
567 correspond to nanoplastics and/or small nanoplastics aggregates were identified both in the
568 control and the treated roots. They were mainly located close to the cell walls of cortex cells.
569 Indeed, plant cells contain various components which may appear similar to nanoparticles (e.g.,
570 polyphosphate granules, small vesicles, etc). While we were not successful in the detection of
571 nanoplastics using nanoCT, we must emphasize that this technique is still promising to
572 eventually detect nanoplastics in complex matrices with further analytical method
573 development. Furthermore, these results bring into question the validity of some of the
574 previously published data regarding the detection of nanoplastics in plant tissues using
575 transmission electron microscopy (TEM). One has to take into account that the use of nanoCT
576 allows scanning over a wide area and the ability to reconstruct multiple sections. This is in
577 contrast to analyzing samples with TEM where only a few thin sections or micrographs are
578 analysed so the probability of having a nanoplastic in any given thin section would be low and
579 the probability of (incorrectly) identifying a structure as a nanoplastic when it is in fact a naturally
580 occurring feature is high. For example, in two recently published manuscripts, the authors
581 speculated that they identified nanoplastics in plant tissues based on TEM images alone. (Dong
582 et al., 2021; Lian et al., 2021) This result is highly questionable since no control TEM images of
583 the plant tissues are shown and therefore there is no clear evidence that the globular structures
584 the authors identified correspond to nanoplastics as opposed to natural structures.

585 These results illustrate the real challenge of identifying nanoplastics particles in plant tissues and
586 reinforces the interest of the approaches we have developed using Pd-doped nanoplastics and
587 Pd measurement by ICP-MS, as well as the need to further develop advanced nanometrology
588 techniques to eventually confidently measure nanoplastics without the aid of a tracer. [The](#)

589 approach used in the present study can be applicable to study other plant species as well as
590 other organisms. I.e Pd-doped nanoplastics have been successfully applied to investigate the
591 transport of nanoplastics in salmon gut sac ex vivo(Clark et al., 2022) and the uptake and
592 egestion of nanoplastics by freshwater amphipod (Redondo-Hasselerharm et al., 2021) and
593 earthworms (Lahive et al., 2022). Other polymers as well as other plastic morphologies with
594 environmental relevance, such as fibers, can be envisaged as well (Frehland et al., 2020; Keller
595 et al., 2020; Lahive et al., 2021; Mitrano et al., 2019).

596

597 **3.4. Towards understanding impacts of nanoplastics exposure to wheat plants**

598 This study highlights the potential of nanoplastics to be absorbed and transported to the aerial
599 parts of wheat. Further studies should be conducted to evaluate the possible transfer of
600 nanoplastics to the grains. In addition, we have shown important impacts on the cell walls
601 (biochemical and structural) of exposed plants that are translated in changes in the root
602 anatomy. Finally, we have shown the role of root exudates and mucilage in determining the
603 behaviour of nanoplastics in the growth media. Further studies on exudation rates and
604 composition for plants exposed to nanoplastics would help to better understand phytotoxicity
605 and tolerance mechanisms in plants and would be a key in understanding the behaviour of
606 nanoplastics in the growth media. This study highlights the need to explore new biomarkers and
607 biological responses when exposed to a new type of pollutant (i.e. nanoplastics in this case). This
608 may be applicable for other biological models and ecotoxicological studies in general, with the
609 ultimate goal of establishing the most appropriate standard end points to allow for the
610 comparison amongst different studies.

611

612 This study successfully demonstrated the potential of doped nanoplastics to trace nanoplastics
613 in a simplified system. Following this proof of concept, further research should head towards
614 more environmentally realistic conditions. First, the use of hydroponic system allowed to
615 decouple the effects of the growth environment from the impacts of particulate plastic itself,
616 but it is not representative of the complex soil environment. Further studies should be
617 conducted to test whether plant uptake and translocation of nanoplastics also occurs in plants
618 grown in soils. Second, concerning the dose, there is insufficient data on nano and microplastics
619 concentrations in agricultural soils, so the method applied in this study should be tested over a
620 wider range of concentration to cover the potential range of concentrations in soils. Third,
621 pristine nanoplastics of only one type of polymer and size and two different morphologies have
622 been tested. In summary, future research on nanoplastics-plant interactions should move to soil
623 and long-term experiments to assess the real risk in terms of trophic transfer and crop
624 production but still aim to understand the mechanisms which are responsible for uptake and
625 impacts. This implies that better categorization of particle characteristics is needed to delineate
626 their role in particle behaviour, i.e. polymer type and presence of additives, size and surface
627 charge and heteroaggregation within the soil matrix. The study of uptake and transport routes
628 in plant and the systematic study of soil characteristics influencing these processes must
629 therefore still be considered. We anticipate that the aging process(es) of nanoplastics in the
630 dynamic plant-soil system in which soil components and plant rhizosphere would strongly
631 determine the fate of nanoplastics.

632 Considering the worldwide context of advancing agricultural practices with an increasing human
633 population, the development of more efficient practices will be paramount to ensure a safe food

634 supply while optimizing resources and minimizing pollution. These developments are expected
635 to strongly rely on the use of plastics both in greenhouses and in the fields, such as mulch films.
636 Further plastic burdens on agricultural fields may be expected with the application of sewage
637 sludge as organic fertilizer. It is therefore urgent to increase our knowledge about the impact of
638 the presence of plastic residues and notably, nano and microplastics in agricultural systems. This
639 study provides a foundation on the applicability of using metal-doped nanoplastics and adapted
640 analytical tools to study the fate of nanoplastics in agricultural systems.

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659

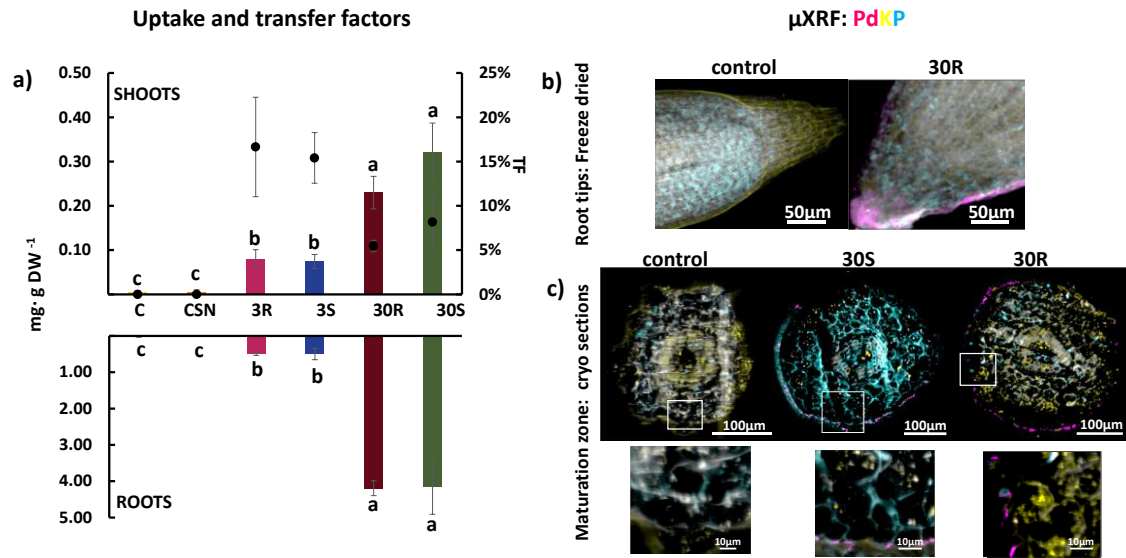
660 **Author contributions**

661 A.E.P.R conceived the study, was involved in the acquisition of μ CT and μ XRF data, participated
662 in the analysis and interpretation of data and wrote the manuscript. D.M.M contributed to the
663 conception of the study, provided the nanoplastics, was involved in the acquisition of μ CT data,
664 performed ICP-MS analysis and the leaching experiment and contributed to the manuscript
665 writing. H.C contributed to the conception of the study, was involved in the acquisition of μ CT
666 and μ XRF data, participated in data analysis and contributed to the manuscript writing. M.W
667 conducted the plant experiment, the analysis of phytotoxicity markers and the acquisition of
668 ATR-FTIR spectra. J.R.H developed the methodology for the analysis of ATR-FTIR. E.B and B.H
669 analysed the μ CT data, wrote the corresponding methods section and revised the manuscript.
670 J.V performed the acquisition of nanoCT data and the 3D reconstructions. G.S conceived the
671 study, supervised the plant experiment, was involved in the acquisition of μ CT data, performed
672 the SEM analysis, and contributed to write the manuscript. All the authors have given approval
673 to the final version of the manuscript.

674

675 **Competing interests**

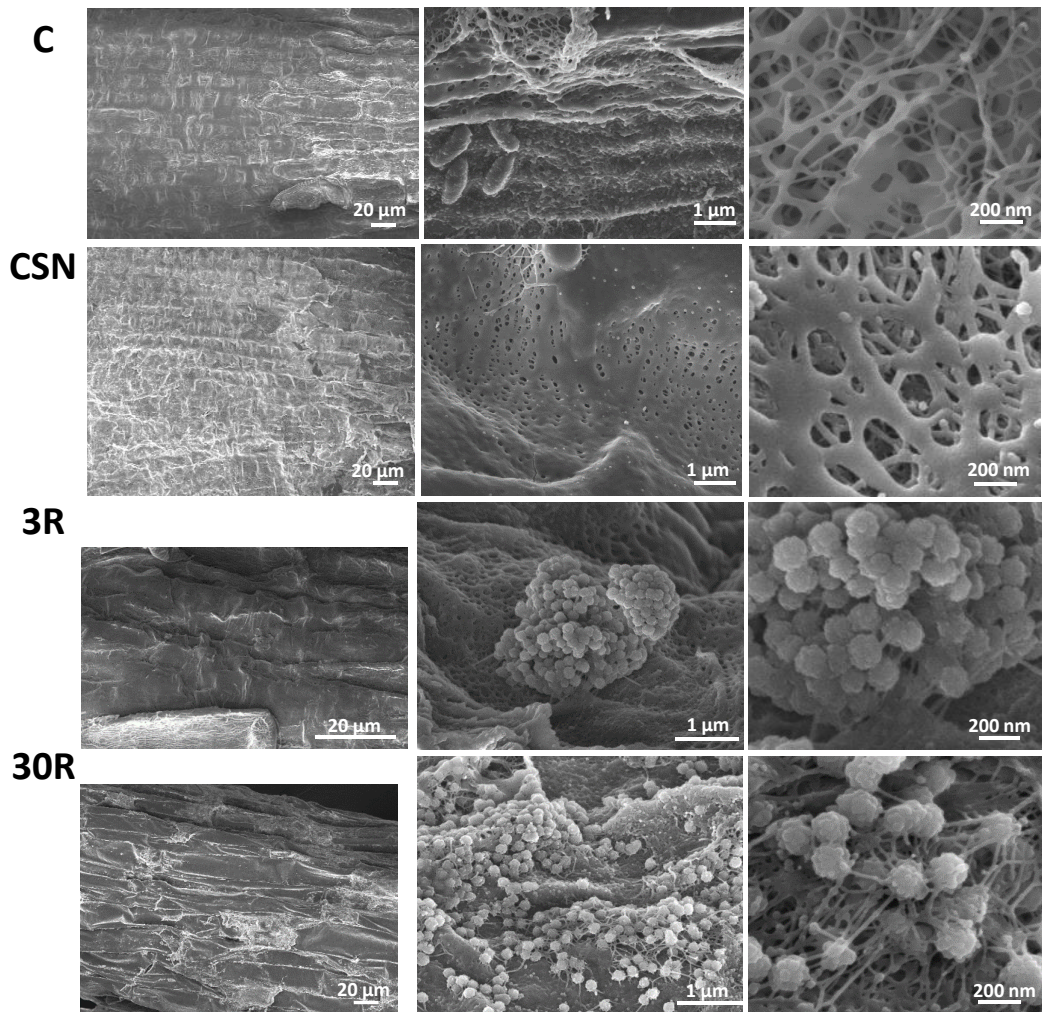
676 The authors declare no competing interests.



678

679 **Figure 1: a) Nanoplastics concentration in shoots and roots. Bars are average nanoplastics concentration (mg·gDW⁻¹) (N=4), error bars are standard deviations. Different letters mean significant differences among treatments (Tukey test at $p \leq 0.05$). Points are transfer factor (%). μ XRF tricolor maps showing the distribution of Pd (pink), K (yellow) and P (blue) of freeze-dried root tips b) and cryogenic root cross section of maturation zone.**

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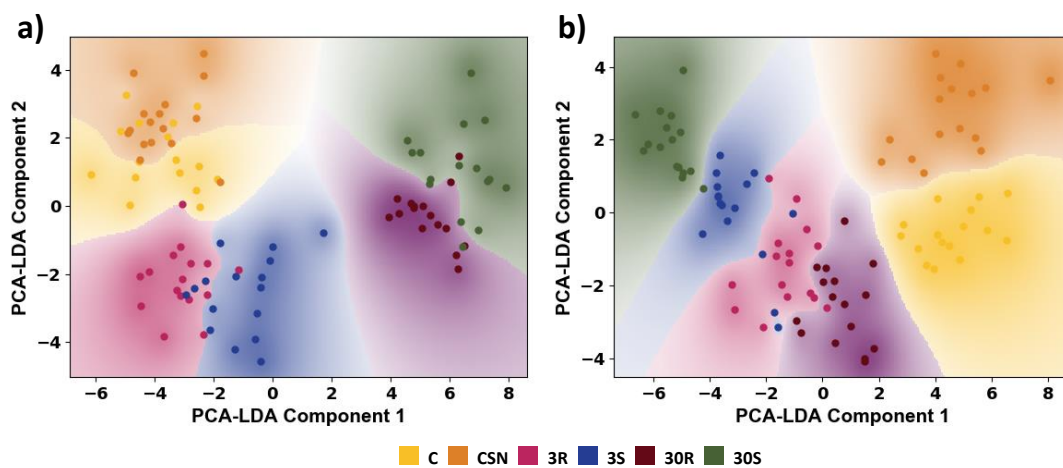
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Figure2: SEM observations of the root surface, 400 μm from the apex, for plants exposed to raspberry nanoplastics. Nanoplastics are agglomerated and associated with polysaccharide filaments.



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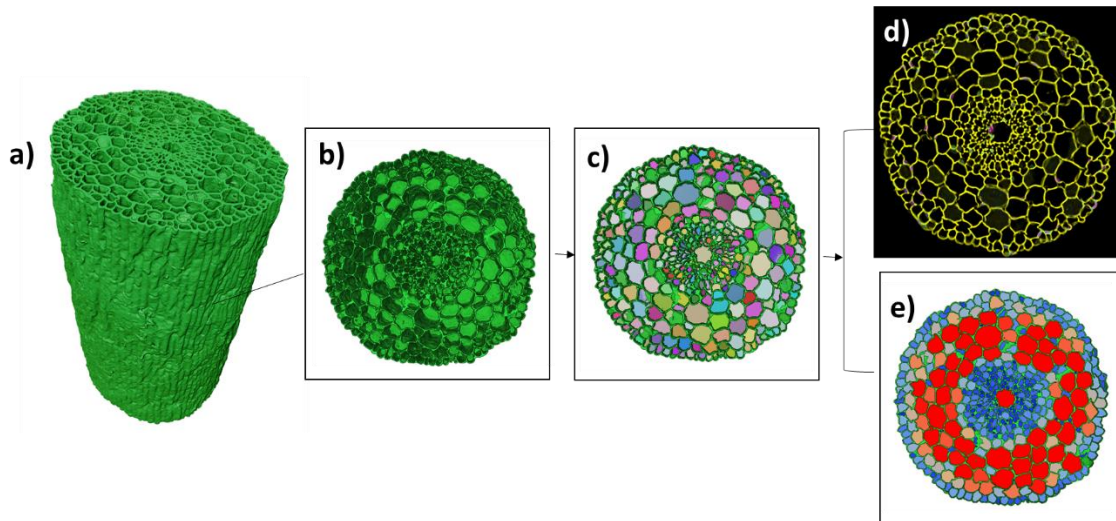
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Figure 3: PCA-LDA of the ATR-FTIR spectra collected from roots a) and shoots b). Four biological replicates per treatment ($n=4$), with four technical replicates performed for each biological sample.

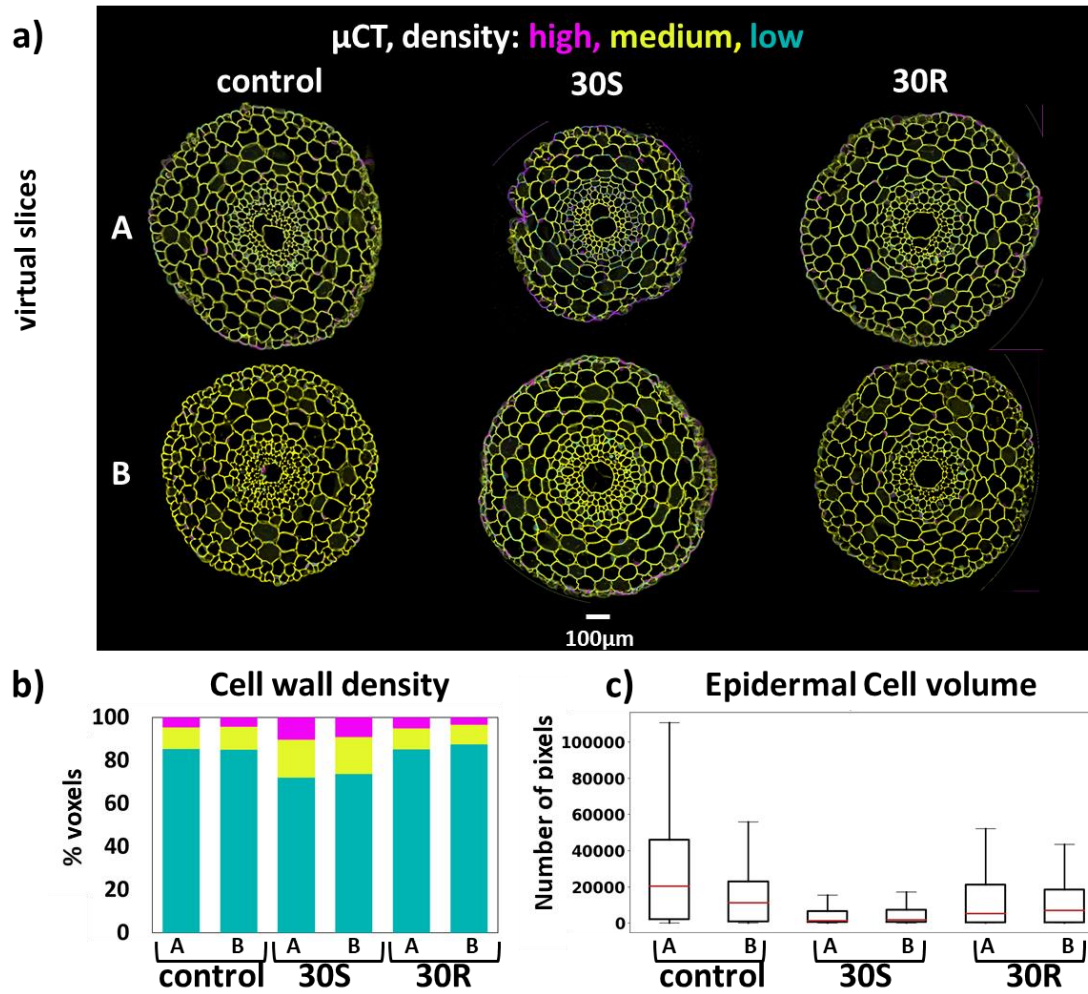


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695 **Figure 4: Impacts of nano-plastic on root architecture studied by synchrotron μ CT with phase contrast: a) 3D**
 696 **rendering showing cell walls, b) rendering along Z-axis of the cell walls, c) virtual section through the 3D volume**
 697 **of the segmented cell walls and the labelled cell volumes, d) virtual slice (average of 20 μ m along Z-axis)**
 698 **through the 3D volume showing the distribution of high (magenta), medium (yellow) and low(cyan) cell wall density**
 699 **regimes, e) virtual slice showing cell walls and color-coded volumes from small (blue) to large(red). Allocations of**
 700 **cells according to regions of interest /epidermis, cortex and stele can be done based on their size and location.**

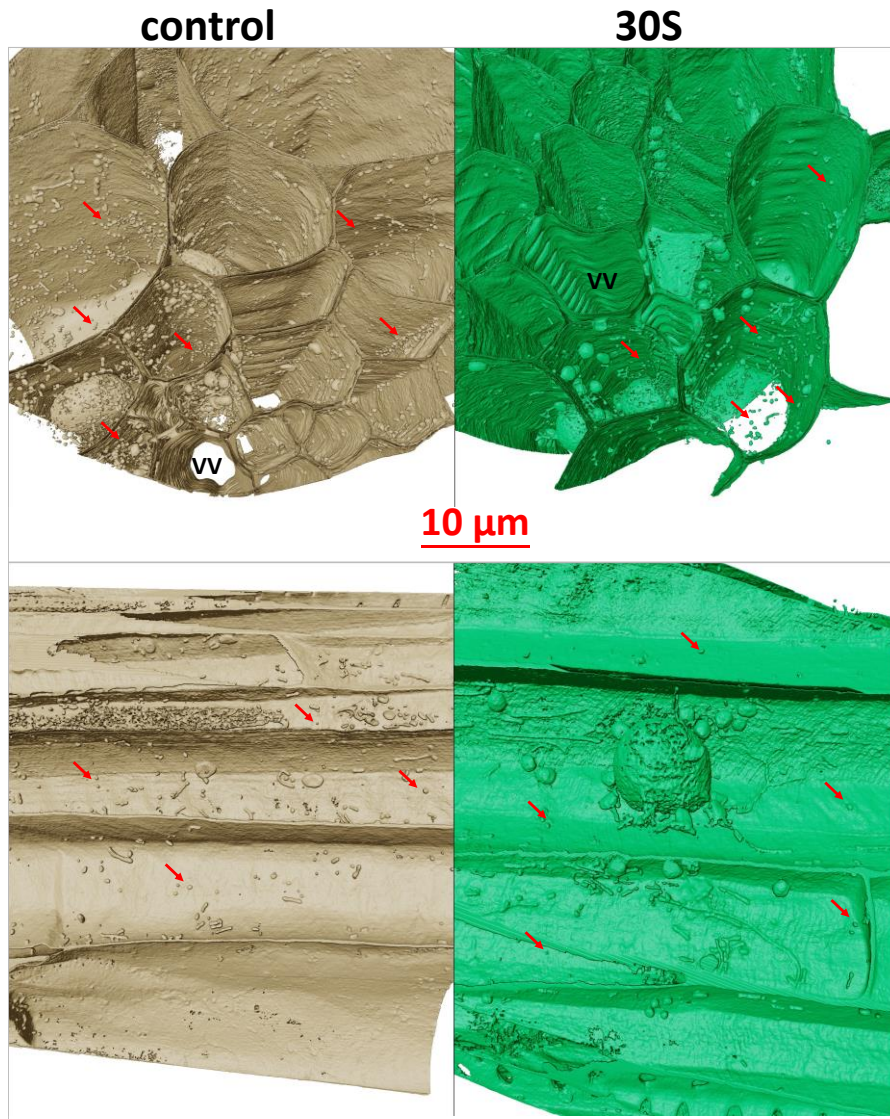
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704 Figure 5. Impacts on root architecture studied by synchrotron μ CT with phase contrast (n=2): a) distribution of high
 705 (magenta), medium (yellow) and low (cyan) cell wall density regimes in a virtual root slice (20 μ m), b) proportion of
 706 voxels in each cell wall density regime, c) boxplot showing epidermal cell volume: box shows interquartile range
 707 (IQR) Q1 to Q3, the red line is the median, whiskers show maximum and minimum (1.5 x IQR), outliers are not
 708 shown.



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711 Figure 6: Analysis of roots by synchrotron nanoCT with phase contrast (n=1). 3D rendering of control root (brown)

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713 and root exposed to 30 mg·L⁻¹ smooth nanoparticles (green) showing cortex cells and vasculature vessels (VV). Red

714 arrows show particle-like structure with size lower than one micron. Full views are shown in Supplementary figure

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Assessing implications of nanoplastics exposure to plants with advanced nanometrology techniques

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Abstract

Despite the increasing attention given to the impacts of nanoplastics in terrestrial environments, there is limited data about the effects on plants, and the quantitative information on uptake. In the present study, wheat plants grown in hydroponics were exposed to Pd-doped nanoplastics. This allowed us to quantify nanoplastics uptake and translocation to the shoots. Visualization of nanoplastics in roots was performed with synchrotron micro X-ray fluorescence (μ XRF). Nanoplastics accumulated on the root epidermis, especially at the root tip and in root maturation zones. A close relationship between plant roots, rhizodeposits and nanoplastics behaviour was shown. Reinforcement of the cell wall in roots was evidenced using Fourier transform infrared spectroscopy (FTIR) and synchrotron-computed microtomography (μ CT). Synchrotron-computed nanotomography (nanoCT) evidenced the presence of globular structures but they could not be identified as nanoplastics since they were observed both in the control and treated roots. By utilizing the inorganic tracer in the doped-nanoplastics, this study paves the road for elucidating interactions in more complex systems by using an integrative approach combining classical phytotoxicity markers with advanced nanometrology techniques.