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Uptake of Polystyrene Nanospheres by Wheat and Arabidopsis Roots in Agar, Hydroponics, and Soil†

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Plant uptake of micro- and nanoplastics can lead to contamination of food with plastic particles and subsequent human consumption of plastics. There is evidence that plant roots can take up micro- and nanoplastics; however, most of this evidence stems from experiments conducted with plants grown in hydroponics or agar systems where uptake of nanoparticles by roots is more favorable than when plants were grown in soil. Here, we discern the root uptake and accumulation of polystyrene nanospheres in plants grown in different growing media: agar, hydroponics, and soil. In addition, we tested the impacts of nanospheres on plant biomass and plant stress. Wheat and *Arabidopsis thaliana* were grown in agar, hydroponics, and soil media and exposed to polystyrene nanospheres. Three different nanospheres were used (40 nm and 200 nm carboxylate-modified and 200 nm amino-modified polystyrene) and uniformly mixed into the growing media. Plants were grown for 7 to 10 days and roots were then examined for the presence of nanospheres by confocal laser scanning microscopy and scanning electron microscopy. Plant stress was evaluated by measuring reactive oxygen species (ROS). We observed the 40 nm nanospheres inside plant roots, but the 200 nm nanospheres only adhered to root cap cells showing no uptake into the roots. Furthermore, confocal images indicated that root uptake of nanospheres was favored in hydroponic solutions as compared to agar and soil media. Plant biomass was generally not affected by the nanospheres, except for hydroponically grown *A. thaliana*, where biomass was significantly reduced. Small sized (40 nm) and positively charged (200 nm amino-modified) nanospheres showed higher ROS accumulation in plants than negatively charged 200 nm carboxylate-modified nanospheres. This study provides evidence that polystyrene nanospheres can be taken up into the interior of plant roots and cause plant stress, but these impacts are less pronounced in media where the plastic particles are less mobile, like in agar and soil media as compared to hydroponic systems.

Environmental significance

Micro- and nanoplastics pose a threat to terrestrial ecosystems because they can impair soil and plant health. Micro- and nanoplastics have been shown to be taken up by plants through roots, and this provides a pathway for human exposure when plants are consumed. Most of the evidence of root uptake of micro- and nanoplastics stems from hydroponic systems; however, when plants are grown in soil media, then uptake of plastic particles by roots is likely less pronounced than in hydroponics because plastic particles attach to soil particles and are less plant available. Our findings provide experimental evidence that this is indeed the case, as less plastic particles were taken up by roots grown in soil as compared to hydroponics. Plants grown in hydroponics thus are more susceptible to plastic particle uptake than plants grown in soil.

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1 Introduction

Agricultural soils receive nano- and microplastics from different sources such as biosolids application, compost amendments, plastic mulching, irrigation water, and atmospheric deposition[1–3]. Nano- and microplastics can be translocated into the root zone by infiltrating water, tillage, or bioturbation[4]. Crops are therefore inevitably exposed to nano- and microplastics present in soils. If nano- and microplastics are taken up by roots and transferred to the edible parts of the plants, then the plastic particles are being introduced into the food chain and subject to human consumption.

Nano- and microplastics can enter plant roots by two main pathways: the apoplastic and symplastic pathway. The apoplastic pathway is the entry of nano- and microplastics through the spaces between root cells, driven by water movement, but the interior of the cells is not penetrated. This pathway is mainly affected by transpiration rate[5,6]. The symplastic pathway involves the transport of plastic particles through plasmodesmata and the entry of the particles into the cytoplasm of the root cells. This transport mechanism is usually considered to have a size exclusion limit of 40 to 50 nm[7]. A third, less common mode of plant uptake is the entry of plastic particles through cracks formed in the root tissue during lateral root emergence, i.e., crack entry mode uptake[5]. After plastics accumulate in the vascular tissue inside plant roots, they can also be translocated to the stem and leaves[8].

Plant uptake of nano- and microplastics has mainly been studied with model spherical polystyrene particles, ranging from 40 to 2000 nm, and having negative or positive surface charge. The experimental conditions mostly involved growing plants in nutrient solutions spiked with polystyrene nano- and microplastics, either in agar or hydroponic systems. Sun et al.[6] observed limited root uptake of positively charged 265 nm polystyrene spheres, but more uptake of negatively charged 200 nm spheres into the epidermal root tissue and the xylem of *Arabidopsis thaliana*. Li et al.[5], also working with hydroponic systems, observed apoplastic transport of negatively charged 200 and 2000 nm polystyrene spheres, but no penetration beyond the Casparian strip in *Triticum aestivum* and *Lactuca sativa*. However, where the Casparian strip was not intact, i.e., where lateral roots emerge from the main root, polystyrene spheres could enter the vascular tissue of the roots and be translocated to the shoot. This uptake via crack entry mode was postulated as a main mechanism for uptake of plastic by roots, allowing particles up to 2000 nm to enter the vascular system[5].

An important factor that affects the root uptake of nano- and microplastic is the surface charge, which determines how the plastic particles interact with plant cells. Plant roots produce mucilage and exudates, which are negatively charged, and which stick to positively charged nano- and microplastics and inhibit penetration into the root[9]. Indeed, the translocation of negatively charged polystyrene nanospheres to different plant parts was more prominent than that of positively charged nanospheres in *A. thaliana* grown in half-strength MS medium[6]. Nonetheless, positively charged polystyrene nanospheres induced oxidative stress, inhibited seedling development, and plant growth[6].

Hydroponic systems have been used in most studies where uptake of polystyrene spheres into roots has been reported[5,6,10–13]. Using TEM imaging, Dong et al.[10] detected 200 and 1000 nm polystyrene nanoparticles in the root cortex of *Daucus carota* L., but only the 200 nm spheres were observed in the stele and xylem. Also using TEM imaging, Spano et al.[11] found that 50 nm polystyrene nanoparticles could penetrate into the cytoplasm and vacuoles of *Oryza sativa* L. Fifty nanometer polystyrene nanoplastics were found to accumulate inside plant roots when grown in vermiculite and half strength Murashige and Skoog (MS) solution[14].

Hydroponic systems are most conducive for root uptake of nano- and microplastics because plastic particles are highly mobile and have frequent contact with plant roots. However, when plants are grown in agar or soil, nano- and microplastics are not as mobile and are not readily available for root uptake. Indeed, no uptake of polystyrene spheres (40 and 1000 nm) was observed when *A. thaliana* and *Triticum aestivum* were grown in agar media; but rather, the polystyrene spheres accumulated at the root cap cells when the roots pushed their way through the agar medium[15]. In real soils, plastic particles are usually attached to soil particles and thus are even less readily available for root uptake.

The overall goal of this study is to assess the interaction and accumulation of polystyrene nano- and microplastics in *A. thaliana* and wheat plants under different growth conditions (agar, hydroponics, and soil). We hypothesized that root uptake of nano- and microplastics depends on the system in which the plants are grown, following the sequence: hydroponics > agar > soil, with limited root uptake in soils. We further tested the effect of plastic size (40 and 200 nm) and surface charge (positive and negative) on the potential root uptake and accumulation on and inside the roots, and whether the plastic particles induce oxidative stress.

2 Materials and Methods

2.1 Plastic materials and characterization

Two different sizes (40 and 200 nm) of yellow-green fluorescent polystyrene nanospheres with two different surface modifications, negatively charged carboxylate- and positively charged amino-modified spheres, were used (Table 1, Figure S1). Excitation and emission wavelengths for the spheres are 505 and 515 nm, respectively. Polystyrene nanospheres, as purchased, were stored in a refrigerator at 4^{o}C. The 200 nm carboxylate- and amino- modified polystyrene microspheres contained sodium azide as a preservative, and to remove the sodium azide, we dialyzed a suspension of 0.29 g/L with a 25 mm 12,000–14,000 MWCO dialysis membrane (Spectra/Por, Spectrum Laboratories, Inc., Rancho Domingues, CA) in deionized water for 3 days. The zeta-potentials of the nanospheres were measured with a Zetasizer (Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) in deionized water and half-strength Murashige and Skoog (MS)

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Table 1 Characteristics of polystyrene nanopheres. Spheres were obtained from ThermoFisher Scientific, USA.

Particle Diameter (nm)	Surface Modification	Color	Excitation (nm)	Emission (peak) (nm)	Zeta Potential† (mV)	Stock Concentration (g/mL)	Lot Nr.
40	carboxylate (-OOH)	yellow-green	505	515	-60.0 ± 2.9	0.05	F8795
200	carboxylate (-OOH)	yellow-green	505	515	-21.4 ± 1.8	0.02	F8811
200	amino ($-NH_2$)	yellow-green	505	515	7.8 ± 4.1	0.02	F8764

† Zeta potential of the spheres measured in distilled water with a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Data are mean and standard deviations of 10 measurements.

solution[16], consisting of macro- and micronutrients and vitamins.

2.2 Plant growth experiments

Plants: Two model plants were used, (i) soft white spring wheat cv. Louise (*Triticum aestivum*), representing a moncot with a fibrous root system, and (ii) *Arabidopsis* (*A. thaliana* ecotype Columbia), representing a dicot with a tap root. The seeds of wheat and *A. thaliana* were washed with 20% bleach and Triton X-100 (Sigma-Aldrich) solution for 10 minutes, followed by rinsing three times with ultra-pure water, then a 70% ethanol solution was added to the seeds for 2 minutes, and finally the seeds were rinsed thoroughly with ultra-pure water[15]. The seeds were then stored in ultra-pure water at 4^oC for three days for cold stratification.

Growth media: Plants were exposed to the nanospheres in three different growth media: agar, hydroponics, and soil. These three media were chosen to represent different uptake scenarios: In agar, nanospheres are immobile, embedded in the semi-solid agar medium, and as such the nanospheres cannot move towards roots, so that root-nanosphere contact can only occur via root interception. In hydroponics, nanospheres are freely mobile and frequent root-nanosphere contact occurs via diffusion and convection (through transpiration). In soil, root-nanosphere contact occurs via a combination of interception and diffusion-convection, superimposed by the interactions of the nanospheres with the soil matrix.

For agar, plants were grown in sterile Petri dishes, for hydroponics, plants were grown in Magenta boxes (PhytoTech Labs, Inc., Lenexa, KS, USA), and for the soil experiments, plants were grown in clay pots and in micro-ROCs (microscopy Rhizosphere Observation Chambers)[17] filled with soil medium (Figures S2, S3). All growth experiments were conducted in a growth chamber. The exposure concentrations for the nanoplastics in all the growth media were chosen as 0.029 g/L or 8.3×10^{11} n/mL for the 40 nm spheres and 0.029 g/L or 6.6×10^9 n/mL for the 200 nm spheres, where the volume refers to the volume of liquid, agar, or soil medium. These concentrations were chosen based on our previous experiments[15] and ensure good visualization of the nanospheres with confocal microscopy.

Agar: For the experiments with agar, the seeds were placed into sterile Petri dishes (Fisherbrand, 08-757-11A or -12, Fisher Scientific) containing 25 mL autoclaved growth medium (half-strength MS solution with 0.7% agar) and fluorescent nanospheres. First, nanospheres were mixed with half-strength MS media (PhytoTech Labs, Lenexa, KS) at the specified concentration (0.029 g/L), and then sonicated for 10 minutes. Agar (Bacto Agar, Becton, Dickinson and Company, Sparks, MD) was added to the mixture. Five and ten seeds of wheat and *A. thaliana* plants, respectively, were used for each experiment. Control samples, containing only seeds with growth medium but no nanospheres, were also prepared. Petri dishes with seeds placed on top of the agar were sealed, and transferred to a growth chamber with growing conditions of a day/night cycle of 16/8 h with temperatures of $22^oC/18^oC$ day/night. Wheat was grown in a growth chamber for 10 days, *A. thaliana* was grown for 7 days. These time periods were chosen based on our previous experience[15], namely that roots were grown sufficiently but not too large for good visualization with confocal microscopy. The plants were then pulled out from the growth medium with a tweezer and placed on a microscopy slide with a few drops of half-strength MS solution, covered with a cover slide, and analyzed with confocal laser scanning microscopy and scanning electron microscopy (SEM). One set of plant roots was washed by dipping the roots into clean half-strength MS solution and moving the roots up and down 6 times. Each treatment was replicated three times, and for each replicate three wheat plants and six *A. thaliana* plants were imaged with confocal microscopy, and three wheat plants were used for root cross-sectioning and SEM imaging.

Hydroponics: For hydroponics, wheat seeds were germinated on autoclaved growth medium (half-strength MS solution with 0.7% agar) in Petri dishes for 2 days, and then the seedlings were transferred to the hydroponic system in a laminar flow hood. For *A. thaliana*, seeds were directly germinated in the hydroponic system. The hydroponic system consisted of a 385 mL Magenta GA-7 tissue culture vessel (Sigma-Aldrich) filled with 200 mL half MS solution for wheat and 100 mL for *A. thaliana*, with and without nanospheres. On top of the hydroponic solutions, we placed a stainless steel mesh (304 Stainless Steel Mesh Screen, ELAFROS, Amazon) which supported the seedlings. The culture vessels were then covered with caps, and placed inside a growth chamber under the same growth conditions as used for the agar media experiments. After 10 days for wheat and 7 days for *A. thaliana*, the plants were removed with a tweezer and then analyzed with confocal laser scanning microscopy and SEM imaging in the same manner as the plants in the agar experiments.

Soil: For the soil medium, an oven dried greenhouse soil mix (professional growing mix, Sun Gro Horticulture, Agawam, MA) was thoroughly mixed with nanoplastics suspension prepared in ultra-pure water using a spatula and glass beaker. The volume of the nanoplastics suspension added to the soil was chosen to obtain a volumetric water content of the soil of 0.25 cm^3/cm^3. The nanoplastic concentrations were chosen such to obtain a final concentration of (0.029 g/L = 2.9×10^{-5} g/cm^3 of soil volume). The plastic-amended soil was packed to a bulk density of 0.31 g/cm^3.

For wheat, soil was packed into clay pots. Wheat seeds were first germinated in Petri dishes with half-strength MS agar media for two days, and then transferred to the clay pots and grown for 10 days. Wheat roots were then gently pulled from the soil and washed with half MS strength media to clean roots for confocal microscopy. The washed plant roots were processed for confocal imaging in the same way as the agar and hydroponically grown plants.

For *A. thaliana*, we observed that the roots got damaged when they were removed from soil medium, and we therefore used a micro-ROC system[17], which allows the roots to grow without direct soil contact. This micro-ROC system has been especially developed to observe root growth by microscopy without having to remove or clean roots[17], and has been used in rhizosphere studies[18]. The micro-ROC system consisted of a 7 cm × 5 cm × 2 cm chamber where one side contained a glass plate and a membrane (38 μm pore size, #400 Nylon cloth, Gilson Company, Inc., Lewis Center, OH) in contact with the soil medium (Figure S3). Mesh and glass slides were attached to the micro-ROC chamber with a sealant (MarineWeld^{TM}, Sulphur Springs, TX). The glass plate of the micro-ROC system was covered with a black plastic film.

The plastic-amended soil was packed into the micro-ROC system at the same bulk density as for the wheat experiments. *A. thaliana* plants were grown such that the roots were confined to the space between the glass slide and the Nylon membrane. *A. thaliana* seeds were first germinated in Petri dishes with half-strength MS agar media for two days, and then transferred to the micro-ROC system and grown for an additional 7 days. The plants were then removed from the micro-ROCs system by removing the glass plate and then prepared for confocal microscopy as described above.

2.3 Confocal laser scanning microscopy

Confocal laser scanning microscopy was performed on a Confocal Microscope (Leica TCS SP8, Leica Microsystems). Microscopy settings are listed in Table S1. Confocal images of the nanospheres in agar medium are shown (Figure S1). The 40 nm nanospheres could not individually be visualized but can be detected as clusters only. For the 200 nm nanospheres, individual particles could be discerned, but a more pronounced signal was obtained with clustered particles.

Before imaging with confocal microscopy, plant roots were stained by dipping the roots in a propidium iodide (PI) staining solution (0.1 mg/mL, Sigma-Aldrich) for 30 seconds. A few drops of half-strength MS solution were placed on a clean microscopy slide, and then PI stained roots were placed onto the slide, and then covered with cover slip with minimal disturbance of the root sections. For both 40 nm and 200 nm spheres, a 488 nm excitation and a 500–550 emission wavelength were used. To show the propidium iodide stained root cells, an excitation wavelength of 561 nm and an emission wavelength of 597–776 nm were used. The magnification used was 10× for wheat and 40× for *A. thaliana*. Imaging was done with z-stacks between the top and the center of the roots in 150–200 and 40–60μm increments for wheat and *A. thaliana*, respectively.

2.4 Scanning electron microscopy (SEM)

Wheat root cross-sections were analyzed with SEM. Wheat roots were stored in a formaldehyde, alcohol, acetic acid solution for 2 weeks, and then cross-sections of 30 μm were prepared with a cryostat (Cryocut 1800 Cryostat, Leica) at -27^oC. Cross-sections were then transferred to SEM stubs and 2% paraformaldehyde was applied for 2 hours. Cross-sections were then frozen in liquid N_2 and freeze-dried overnight. Samples were finally gold-coated and analyzed by SEM (FEI Quanta 200F). Per treatment, three plants were used for SEM, and for each root 10 to 12 cross-sections were analyzed.

2.5 Plant biomass measurements

To determine the effects of plastic particles on plant growth, *A. thaliana* (20 days grown) and wheat plants (10 days grown) in agar and hydroponics were processes for biomass measurements by drying leaves and roots separately. Dry biomass was determined after drying the plants an oven at 70^o for three days. Six plants for *A. thaliana* and three plants for wheat were grown in Magenta boxes per treatment and the experiments replicated three times (Figure S4).

2.6 Reactive Oxygen Species (ROS) measurements

To measure the hydrogen peroxide and superoxide accumulation in plant roots and leaves, the protocol developed by Bittner et al.[19] was followed. Seedlings (7 days old) and leaves (20 days old) from *A. thaliana* were processed for ROS measurements. First, *A. thaliana* seedlings in hydroponics were stained overnight with DAB (3,3-Diaminobenzidine) for hydrogen peroxide accumulation and NBT (Nitroblue Tetrazolium) for superoxide accumulation. After staining, stained seedlings and leaves were washed with a de-staining solution consisting of ethanol, acetic acid, and glycerol in a hot water bath at 60^oC. After the de-staining procedure when the plants looked almost white, the plants were imaged with a fluorescence microscope (LEICA M2O5 FA, Leica Microsystems).

The images obtained from the fluoresence microscope were analyzed with ImageJ[20]. The original color images (RBG format) were converted to a grey scale with a 32-bit format. These black and white pictures were then used to quantify the area of the original coloration (red for DAB and blue for NBT, respectively)[19].

2.7 Quality assurance and quality control

All the materials used for plant growth experiments were sterilized to ensure no microbial growth in growth media and plant roots themselves. Sterilization was done by autoclaving the growth media at 121^oC for 50 minutes, whereas the plant seeds were sterilized by 20% bleach and Triton X-100, and ethanol as

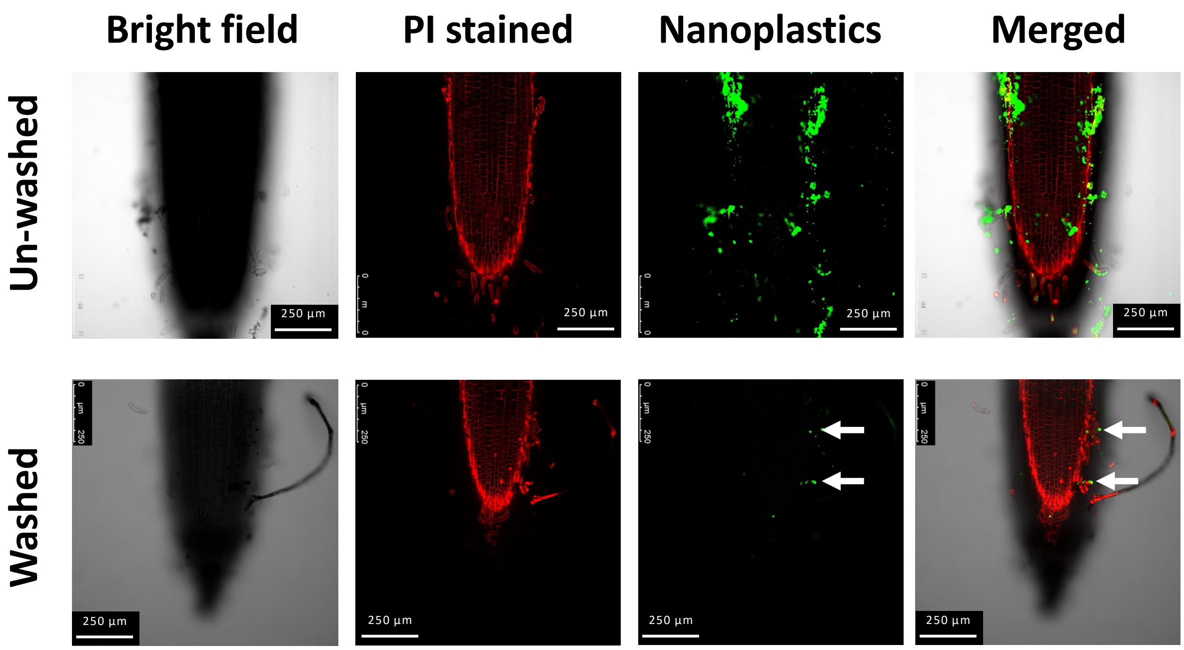

Fig. 1 Confocal images of wheat roots (un-washed versus washed) grown in hydroponic solution with 200 nm carboxylate-modified polystyrene spheres. Arrow indicate nanospheres attached to the root surface after washing. PI: propidium iodide.

mentioned in the agar and hydroponics methods section. Seed sterilization and all sample processing tasks were done in the laminar flow hood to limit ambient plastics contamination. Control treatments with no plastics were included along with the plastic treatments for all experiments. For washing the seedlings before confocal imaging, autoclaved half strength MS solutions were used.

2.8 Statistical analysis

Statistical analysis was done in RStudio to test for differences in shoot and root biomass of the different treatments. ANOVA and Tukey HSD tests were used to compare means of different treatments and to determine the p-values. Statistical significance was considered at the $p < 0.05$ level.

3 Results and Discussion

In the following, we will discuss the associations of the nanospheres with the wheat and *A. thaliana* roots for agar, hydroponics, and soil media. We will then compare the effects of nanosphere size and surface charge. Finally, we will discuss the impacts of the nanospheres on biomass and plant stress.

3.1 Washed versus non-washed roots

The gentle washing by immersing the roots in clean half MS solution removed a substantial amount of nanospheres associated with the roots (Figure 1). This indicates that a large portion of the nanospheres were loosely attached and not internalized into the root tissue. A small fraction of the spheres, however, remained attached to the root epidermis after washing.

3.2 Nanoplastics association with plants grown in agar medium

Wheat: Confocal images of wheat roots indicate that the different types of nanospheres visible in the images were located at the surface of the roots (Figure 2, left and Figure S5). No nanosphere fluorescence could be detected inside the root tissue, as evidenced when examining the z-stacks, which indicate fluorescence along root hairs and the perimeter of the roots only (Figure S5).

Scanning electron microscopy images, however, indicate that the 40 nm carboxylate-modified spheres could penetrate into the wheat roots (Figure 3, left). The 40 nm spheres were detected in the vascular system of the roots, but there was no evidence for the presence of the 200 nm carboxylate- and amino-modified spheres in either the cortex or the vascular tissue. Only a few 40-nm spheres were detected in the vascular tissue, which explains why the confocal images did not show these spheres, as the confocal microscope resolution was not sufficient to resolve individual 40-nm spheres.

Arabidopsis thaliana: The confocal images of agar grown *A. thaliana* indicate no distinct fluorescence inside the roots (Figure 2, right). The nanoplastics were adhered to the outer surface of the roots in aggregates. The z-stack images also show no conclusive evidence of nanoplastics entry into the root's interior (Figure S6).

Taylor et al.[15] also did not find evidence for root entry of 40 nm polystyrene nanoplastics for agar grown *A. thaliana*; confocal imaging only indicated that nanoplastics attached to the root cap cells. On the other hand, Parkinson et al.[21], also using confocal imaging, found that negatively charged 50 nm polystyrene spheres were taken up by agar grown *A. thaliana*; however, the concentrations of the polystyrene spheres used was about 35

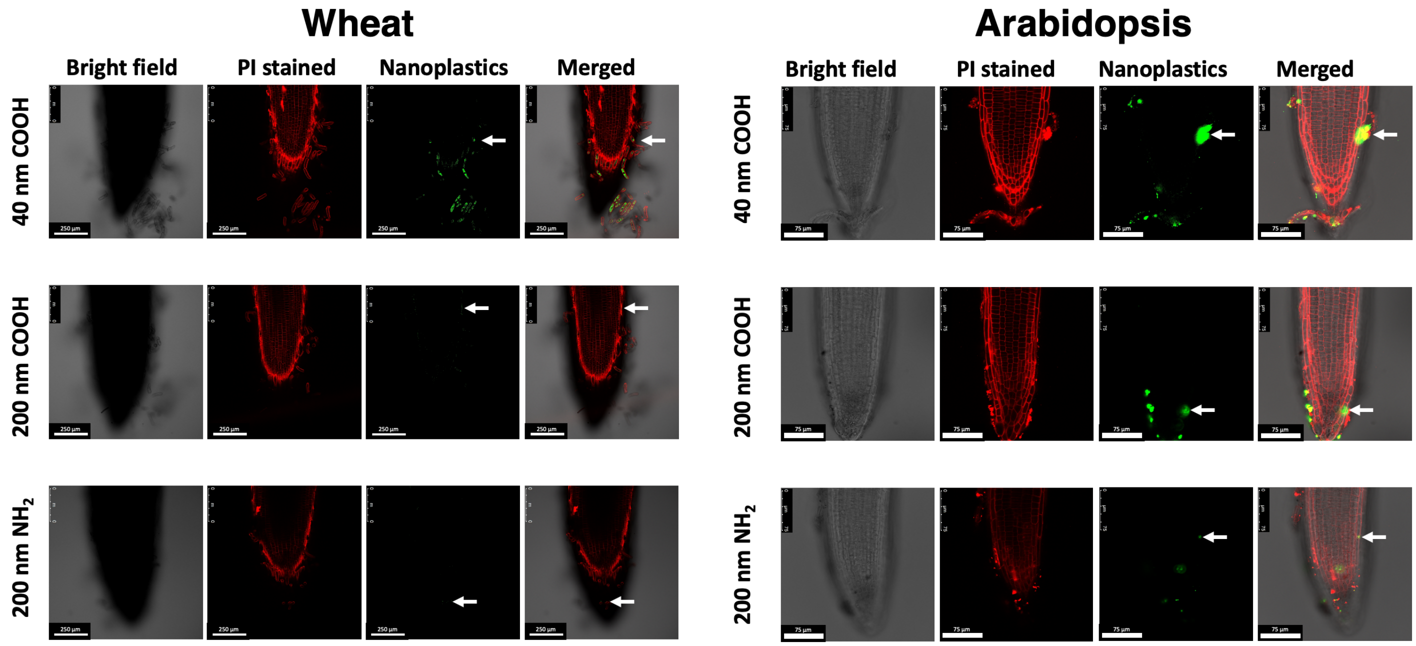

Fig. 2 Wheat (left) and *Arabidopsis thaliana* (right) in agar medium: Confocal images of roots for 40 nm carboxylate-modified polystyrene nanospheres, 200 nm carboxylate-modified polystyrene nanospheres, and 200 nm amino-modified polystyrene nanospheres. The images were focused about 225 μm above the center of the root. Arrows indicate nanospheres attached to the root cap cells. PI: propidium iodide.

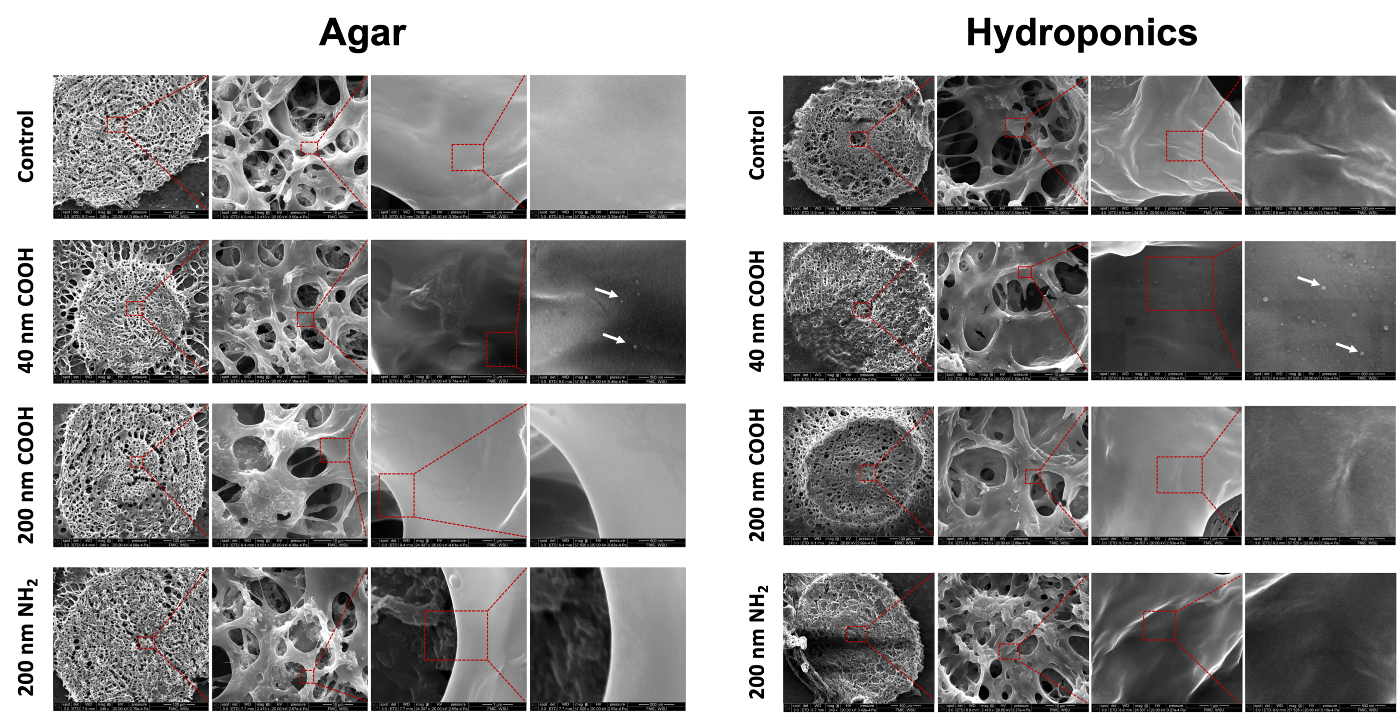

Fig. 3 Scanning electron microscopy images of wheat root cross-sections in agar (left) and hydroponics (right): Ccontrol (no spheres), 40 nm carboxylate-modified polystyrene nanospheres, 200 nm carboxylate-modified polystyrene nanospheres, and 200 nm amino-modified polystyrene nanospheres. Images show different magnifications as indicated by the red boxes. Cross-sections shown were taken about 1 mm from the root tip. Arrows indicate nanospheres inside the vascular system, detected only for the 40 nm spheres.

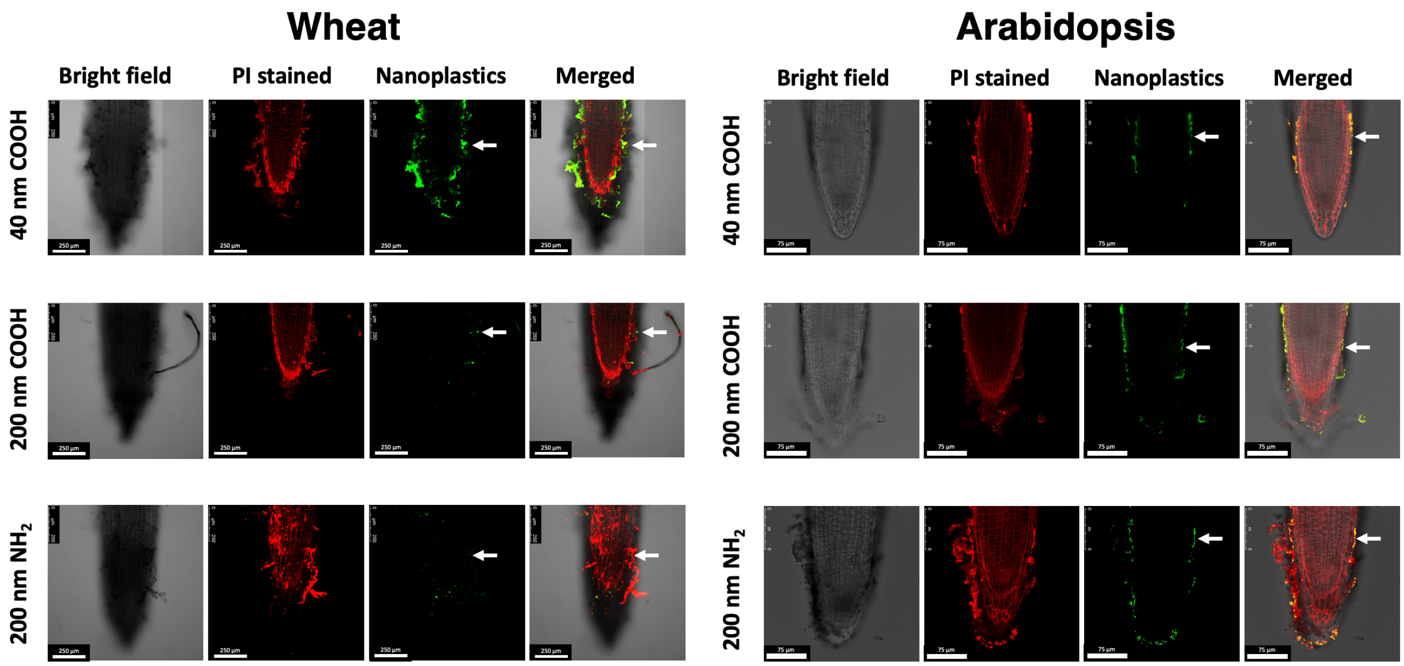

Fig. 4 Wheat (left) and *Arabidopsis thaliana* (right) in hydroponics medium: Confocal images of roots for 40 nm carboxylate-modified polystyrene nanospheres, 200 nm carboxylate-modified polystyrene nanospheres, and 200 nm amino-modified polystyrene nanospheres. The images were focused about 225 μm above the center of the root. Arrows indicate nanospheres attached to the root cap cells. PI: propidium iodide.

times larger.

3.3 Nanoplastics association with plants grown in hydroponic system

Wheat: In hydroponic solutions, the nanospheres were associated mainly at the surface, i.e., epidermis, of the wheat roots (Figure 4, left and Figure S7), irrespective of size or surface charge. Confocal images of wheat root tips did not reveal conclusively whether spheres were taken up into the interior of the roots. The *z*-stack images rather indicate that the spheres accumulated at the root surface as the fluorescence of the spheres was mostly confined to outside cross-section in each *z*-stack. As the focal plane of the microscope was moved from top to the center of the root, the fluorescent signal of the nanospheres remained mainly at the outline of the root (Figure S7).

The strongest fluorescent signal from the nanospheres was obtained with the 40 nm nanospheres, a much weaker signal was detected for the 200 nm spheres (Figure 4, left), indicating that the 40 nm nanospheres were more strongly attached to the root surface than the 200 nm spheres, which could more readily be washed off by gentle rinsing of the roots (Figure 1).

The SEM images of the wheat root cross-sections are shown in Figure 3 (right). Spheres were detected inside the roots for the 40 nm polystyrene spheres treatment; no 200 nm spheres were detected inside the root cross-sections. We detected the 40 nm spheres in the vascular system, indicating that the 40 nm spheres were able to penetrate into the center of the roots and were able to pass through the Casparian strip.

Arabidopsis thaliana: The confocal images indicate that the nanospheres were mostly confined to the exterior of the roots, with the fluorescence of the spheres only visible at the outermost cell layers, i.e., the epidermis (Figure 4, right). This was confirmed by the *z*-stack images, which show that the sphere fluorescence remained at the outline of the root cross-sections as the focal plane of the microscope moved through the *z*-direction of the root tip (Figure S8).

For the amino-modified nanospheres, we observed extensive mucilage around the root tips (Figure 4, right and Figure S8). The nanospheres were mainly located in this mucilage layer. Considerably more fluorescence around the roots was observed for the hydroponics system as compared to the agar system.

3.4 Nanoplastics association with plants grown in soil

For soil grown wheat seedlings, the fluorescence of the spheres was considerably lower as compared to agar and hydroponics systems, and fewer nanospheres were attached to root caps (Figure 5, left and Figure S9). This is because, in the presence of soil, the exposure of plant roots to nanoplastics is limited. Plant roots can access readily available nanoplastics in soil pore water, whereas nanoplastics sorbed to soil particles and organic matter are only available after desorption[22] or root interception. In the case of *A. thaliana*, where the roots were not directly in contact with the soil particles, the nanospheres were still accessible to plant roots as we observed fluorescent nanospheres attached to the root surfaces (Figure 5, right). However, the fluorescence was considerably less pronounced than in agar and hydroponics systems.

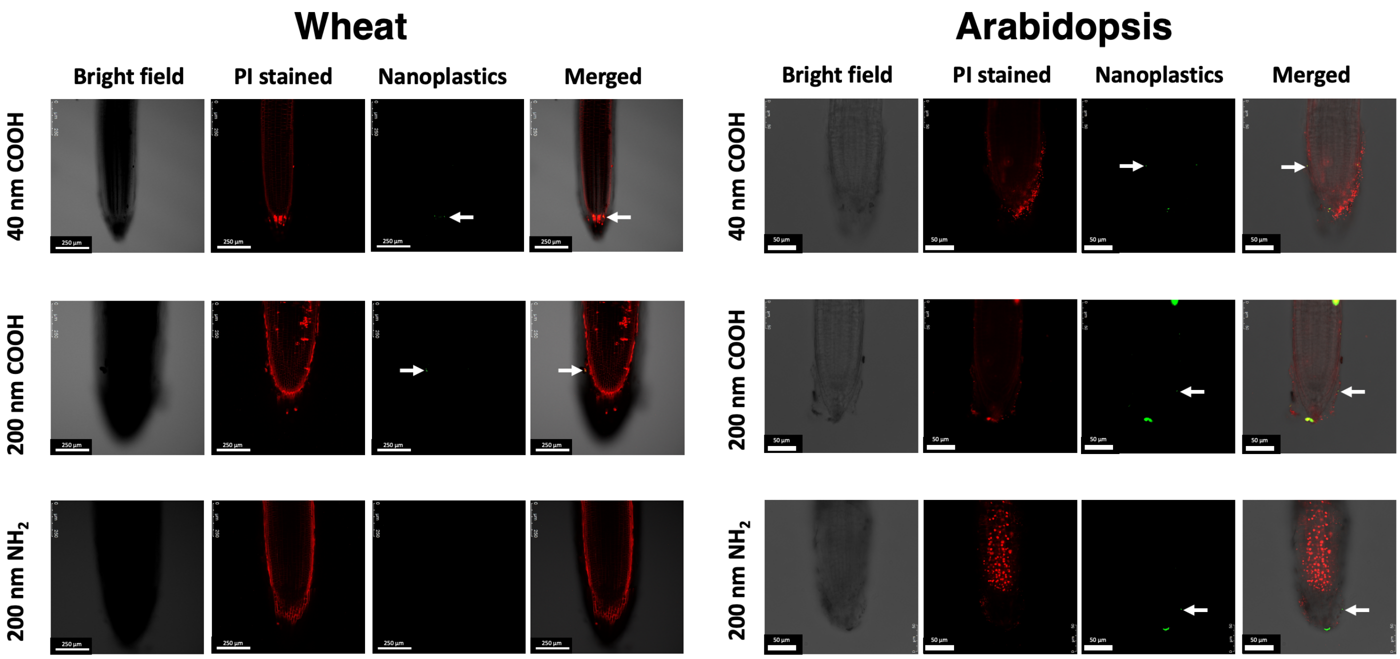

Fig. 5 Wheat (left) and *Arabidopsis thaliana* (right) in soil medium: Confocal images of roots for 40 nm carboxylate-modified polystyrene nanospheres, 200 nm carboxylate-modified polystyrene nanospheres, and 200 nm amino-modified polystyrene nanospheres. The images were focused about 225 μm above the center of the root. Arrows indicate nanospheres attached to the root cap cells. PI: propidium iodide.

3.5 Mechanisms and pathways of nanoplastic uptake by roots

A higher number of nanospheres were associated with plant roots grown in hydroponics as compared to agar and soil. Hydroponics systems offer more opportunity for plant roots to be exposed to nanoparticles because the particles are more mobile and therefore more readily bioavailable than in agar and soil[23,24]. In agar, nanospheres are immobile and can only interact with plant roots when the roots make direct contact with the nanospheres, so the probability of nanospheres-root contact is much smaller in an agar system as compared to a hydroponics system.

In soil, the mobility of nanospheres depends on various factors, such as the size and surface charge of the spheres, and the tortuosity and connectivity of the flow pathways. Furthermore, positively charged nanospheres will be attached to the generally negatively charged soil particles by electrostatic forces, and will therefore only interact with roots through direct contact when a root intercepts a nanosphere during root growth. Negatively charged nanospheres, on the other hand, can be mobile and can move to the root surface via diffusion and convective water flow driven by transpiration. Nonetheless, attachment of negatively charged nanospheres still can occur through pore straining, water film straining, wedging, attachment to the air-water interface or the air-water solid triple point[4]. These mechanisms make nanospheres in soils generally less mobile than in hydroponics systems[25].

Further, plants grown in hydroponics systems have higher transpiration rates which facilitates nanoplastics uptake as compared to soils[23]. Li et al.[5] observed higher uptake and toxicity of 200 nm polystyrene nanoplastics by plant roots grown in hydroponics whereas no uptake was seen in soil grown plant roots. In our study, we did not observe intracellular accumulations in hydroponics, agar, or soil; however, extracellular accumulation and attachment of nanospheres was higher in hydroponics as compared to soils likely due to higher transpiration rate and higher mobility of nanospheres in hydroponics systems.

We did not observe a notable difference between differently charged polystyrene nanospheres, i.e., 200 nm COOH-modified (negative, -21.4 ± 1.8 mV) and 200 nm NH_2-modified (positive, -7.8 ± 4.1 mV) (Table 1), in terms of their association with plant roots. Sun et al.[6] showed higher uptake of negatively charged 200 nm nanospheres than of positively charged ones. However, in our study, confocal and electron microscopy analysis did not show visual differences in the association of differently charged nanospheres. This contradictory result between these two studies may be due to higher surface charges of the nanospheres used in the Sun et al.[6] study, where the carboxylate-modified spheres had a zeta potential of -53.7 mV (vs -21.4 mV in our study) and the amine-modified spheres had a zeta potential of $+28.1$ mV (vs $+7.8$ mV in our study).

We observed that higher numbers of small sized (40 nm) nanospheres were attached to root cap cells as compared to bigger nanospheres (200 nm) (Figures 2 and 4), and only the 40 nm spheres were detected in the interior of the stele by SEM. Smaller sized nanospheres are more prone to root uptake because of size exclusion limits and chemical and physiological barriers in both the symplastic and apoplastic uptake pathways[10,26,27]. Data compiled in a recent review[28] suggest that only particles of size

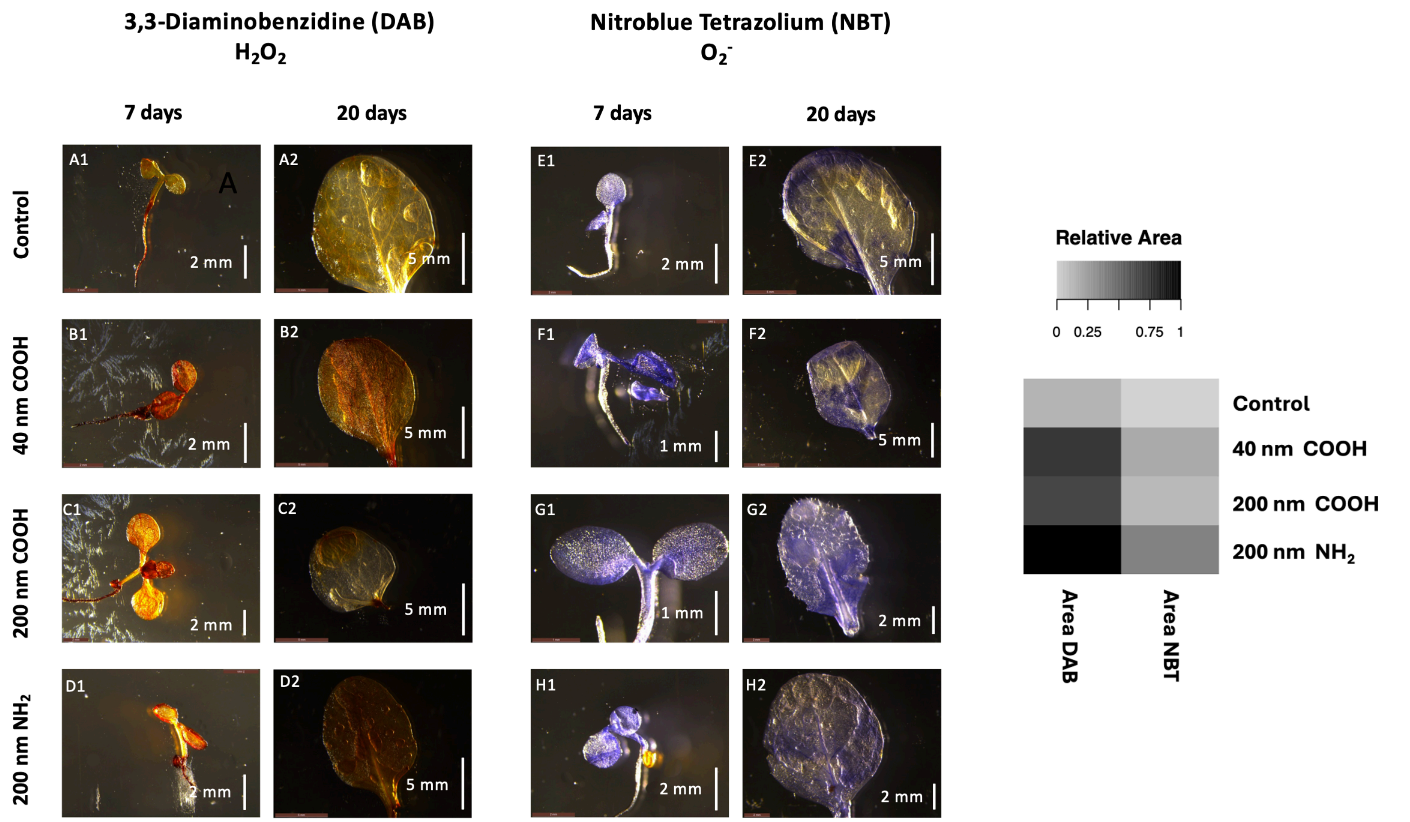

Fig. 6 Fluorescent images (left) and heatmap (right) showing reactive oxygen species (ROS) accumulation in *Arabidopsis thaliana* in hydroponics. Hydrogen peroxide accumulation is shown by red coloration in 7 days grown whole seedlings and in 20 days grown leaves (A,B,C,D). Superoxide accumulation is shown by blue coloration in 7 days grown whole seedlings and in 20 days grown leaves (E,F,G,H). Heatmap shows ROS accumulation on 20 days grown A. thaliana leaves as quantified using ImageJ based on area of brown (DAB) and blue (NBT) coloration. The darker the shading, the greater is the area of coloration, indicating greater ROS accumulation. The statistical differences among the different treatments are listed in Table S3.

of <50 nm can be taken up by roots into the vascular system, whereas particles of size of >100 nm remain on the epidermis. The 40 nm nanospheres in our experiments were likely absorbed by the roots via the apoplastic pathway, and then could penetrate the Casparian strip to enter into the vascular system, as evidenced by the detection of the nanospheres in the stele by SEM.

3.6 Effects of nanoplastics on plant biomass

Wheat and *A. thaliana* plants grown in agar and hydroponics systems with nanoplastics generally showed reduced biomass compared to the control (Figure S4 and Figure S10). The root-to-shoot biomass ratio was also higher for the control than for the nanoplastic treatments (Table S2), indicating that the nanoplastics did interfere with root growth. Sun et al.[6] also observed a reduction in root length when *A. thaliana* was exposed to polystyrene nanospheres at three different concentrations (10, 50, and 100 μg/mL) in half strength MS agar media.

The largest reduction in plant biomass was observed for both wheat and *A. thaliana* for the 40 nm nanospheres in hydroponics systems (Figure S10 and Table S2). This reduction in biomass in hydroponics can be explained by more frequent interactions of plant roots with nanoplastics because the nanoplastic particles can move freely, exposing the plant roots to more nanoplastics than in agar.

In addition, the root and shoot biomass of *A. thaliana* exposed to 40 nm carboxylate-modified and 200 nm amino-modified spheres was significantly lower than that of *A. thaliana* exposed to 200 nm carboxylate-modified spheres. Some of the 40 nm carboxylate-modified spheres were taken up into the vascular tissue of the roots, likely causing the observed reduction in biomass. The 200 nm amino-modified spheres were not detected in the vascular tissue and, like the 200 nm carboxylate-modified spheres, remained concentrated along the epidermis cells. However, due to their positive charge, the amino-modified spheres interact more strongly with the root cells, and thereby can cause more harm to root growth. This is corroborated with the observed higher levels of ROS induced by the amino-modified spheres as discussed below.

3.7 Effect of nanoplastics on reactive oxygen (ROS) generation

To evaluate whether the reduced biomass of *A. thaliana* in hydroponics was associated with an increased concentration of ROS, we assessed the amount of ROS with DAB (3,3-diaminobenzidine) and NBT (nitrioblue tetrazolium). We observed ROS accumulation in *A. thaliana* seedlings and leaves, as indicated by red (H_2O_2) and blue (O_2^-) coloration (Figure 6, left). Qualitatively, nanosphere treatments produced higher ROS accumulation compared to control plants, as indicated by the more intensive coloration (Figure 6, left). Semi-quantitative analysis showed that the 200 nm amino-modified spheres produced more ROS accumulation as compared to 40 nm and 200 nm carboxylate-modified spheres (Figure 6, right).

We also observed effects of nanoparticle size and surface charge on ROS accumulation: negatively charged 40 nm and positively charged 200 nm spheres yielded higher ROS accumulation as compared to negatively charged 200 nm spheres (Figure 6, right). Sun et al.[6] also reported higher H_2O_2 accumulation in *A. thaliana* exposed to positively charged polystyrene nanospheres compared to negatively charged nanospheres. In other studies on duckweed and dandelion exposed to nanospheres, the effect of positively charged nanospheres on ROS accumulation was more pronounced than those of negatively charged nanospheres[29,30]. Positively charged nanospheres exhibit a tendency to undergo heteroaggregation when interacting with negatively charged mucilages and exudates excreted by plant roots[6,31]. This phenomenon of heteroaggregation can lead to the obstruction of root pores, thereby reducing the uptake of water and nutrients by plant roots. Consequently, this obstruction may facilitate the generation of ROS. The size-specific effects observed are attributed to the high surface area of small-sized nanospheres, which promote increased interaction with plant roots and subsequently contribute to the accumulation of ROS.

Generally, plants produce ROS as a response to stress, such as exposure to pathogens[32,33]. The elevated level of ROS when *A. thaliana* was exposed to plastic nanospheres indicates that the plants recognized the plastic nanospheres as a stressor. If ROS levels get too high, then oxidative damage to plant cells can occur[34,35] and plant growth and yield can be negatively impacted[36]. In our experiments, we observed elevated levels of ROS for all plastic treatments, but we did not observe visual cell damage or chlorosis. However, for the negatively charged 40 nm and positively charged 200 nm spheres we observed a reduced shoot and root biomass, indicating that the ROS levels were high enough to cause a phenomenological response, likely a result of reduced photosynthetic activity[34,37].

4 Implications

Micro- and nanoplastics in soil can associate with plant roots in soil, with small nanoplastics (40 nm in our study) taken up by plant roots and translocated into the vascular system of the plants. Larger plastic particles (200 nm in our study) could not penetrate into the vascular system, but nonetheless could attach to the root epidermis. Nanoplastics can have negative impacts on root growth, impacting root and shoot biomass, indicating long-term environmental fate and ecological consequences of plastic particles.

However, most evidence about plant uptake of micro- and nanoplastics stems from experiments with model plastic beads, i.e., polystyrene spheres, and with plants grown in hydroponics or agar systems. While such studies help to elucidate the mechanisms of plastic uptake by plant roots and show that plastic particles can be internalized by roots, we can not conclude from such studies that plastic particles in field soils are indeed taken up by plants also. In our study, we found less plant uptake of nanospheres in soils compared to agar and hydroponics systems, indicating that plastic uptake in field soils is less pertinent than in agar or hydroponics systems. Plastics found in field soils come in many different and irregular shapes, have considerable surface roughness, and can be attached to soil particles, making them less mobile[4]. These attributes make environmentally weathered plastics in soils less plant available compared to model spheres in hydroponics or agar systems. Further, environmentally relevant plastic concentrations in soils are often much lower than those used in experimental studies[38], making plant uptake of plastic particles less likely.

Nonetheless, plastic pollution of soils leads to the possibility of contamination of plant-based products with micro- and nanoplastics and subsequent human exposure to micro- and nanoplastics through food consumption. Recent evidence of the existence of micro- and nanoparticles in the human blood stream[39–42] shows that micro- and nanoparticles reach places where they should not be. The human health impacts of the consumption of micro- and nanoparticles still needs to be evaluated, and steps should be taken to limit direct exposure of humans to plastic by consumption of plastic-contaminated food.

Author Contributions

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Visualization: Kaushik Adhikari
Writing—review & editing: all co-authors

Conflicts of interest

There are no conflicts to declare.

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Uptake of Polystyrene Nanospheres by Wheat and Arabidopsis Roots in Agar, Hydroponics, and Soil†

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Data Availability Statement

All data, including figures and tables, are included in the printed version of the paper.

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† Electronic Supplementary Information (ESI) available: Details on confocal microscopy settings, biomass measurements, statistics, images of polystyrene nanospheres, experimental setups, and confocal *z*-stack images. See DOI: 00.0000/00000000.