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Nanomaterials biotransformation: In planta mechanisms of action.

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(Article begins on next page)

21 April 2025

# Environmental Pollution

## Nanomaterials biotransformation: In planta mechanisms of action

--Manuscript Draft--

<b>Manuscript Number:</b>	ENVPOL-D-22-05734R1
<b>Article Type:</b>	VSI:Pollutants and plants
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<b>Abstract:</b>	<p>Research on engineered nanomaterials (ENMs) exposure has continued to expand rapidly, with a focus on uncovering the underlying mechanisms. The EU largely limits the number and the type of organisms that can be used for experimental testing through the 3R normative. There are different routes through which ENMs can enter the soil-plant system: this includes the agricultural application of sewage sludges, and the distribution of nano-enabled agrochemicals. However, a thorough understanding of the physiological and molecular implications of ENMs dispersion and chronic low-dose exposure remains elusive, thus requiring new evidence and a more mechanistic overview of pathways and major effectors involved in plants. Plants can offer a reliable alternative to conventional model systems to elucidate the concept of ENM biotransformation within tissues and organs, as a crucial step in understanding the mechanisms of ENM-organism interaction. To facilitate the understanding of the physico-chemical forms involved in plant response, synchrotron-based techniques have added new potential perspectives in studying the interactions between ENMs and biota. These techniques are providing new insights on the interactions between ENMs and biomolecules. The present review discusses the principal outcomes for ENMs after intake by plants, including possible routes of biotransformation which make their final fate less uncertain, and therefore require further investigation.</p>
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**Response to Reviewers:**

Please see the attached file for complete Revisions.

Editor and Reviewer comments:

Reviewer #1: Manuscript review of ENVPOL-D-22-05734 "Nanomaterials biotransformation: In planta mechanisms of action"

This review article summarized recent works on the nanomaterial biotransformation within plant tissues using synchrotron-based techniques. The impacts of engineered nanomaterials (ENMs) on plant gene expression were also included. Specifically, studies regarding the biointeraction between plants and ENMs (nanoscale CeO<sub>2</sub>, La<sub>2</sub>O<sub>3</sub>, TiO<sub>2</sub>, Au, FeOx, ZVI, ZnO, CuO, and CdS QDs) were listed and discussed in detail. This review paper contributes to the deep understanding of the fate of ENMs in plant tissues, and the related genetic regulation of plants induced by ENM exposures. The contents are within the scope of the Environmental Pollution. However, I have a number of general concerns, followed by a range of specific comments, which prevent me from recommending this paper for publication in its current form.

General Comments-

Some parts of the abstract and introduction are not quite relevant to the main topic, and need to be revised. The main topic, synchrotron-based analysis of ENMs biotransformation, should be more emphasized and discussed in more detail. In part 1, there was only one sub-title 1.1. None of the figures or the tables provided any information related to the in planta biotransformation. Several figures with summarized information instead of only one figure are better for a review article. Some references are too old or not representative. They should be up to date.

We thank Rev1 for the constructive comments and suggestions given. The manuscript has been thoroughly improved in order to answer to all comments reported. Paragraph subdivision has been updated. Several new references and an additional Figure have been included. All the edited sections have been reported in the answer to each specific question.

Specific Comments-

The abstract used a large amount of space describing exposure pathways and low-dose effects. However, they were not well discussed in the main text. Similarly, the "potential applicability" was not given in the main text. The abstract should contain the most significant findings, critical comments on the current studies, or perspectives for future research.

The abstract has been modified in order to be more informative on the points discussed in the main text. The abstract now reads as follows:

"Research on engineered nanomaterials (ENMs) exposure has continued to expand rapidly, with a focus on uncovering the underlying mechanisms. The EU largely limits the number and the type of organisms that can be used for experimental testing through the 3R normative. There are different routes through which ENMs can enter the soil-plant system: this includes the agricultural application of sewage sludges, and the distribution of nano-enabled agrochemicals. However, a thorough understanding of the physiological and molecular implications of ENMs dispersion and chronic low-dose exposure remains elusive, thus requiring new evidence and a more mechanistic overview of pathways and major effectors involved in plants. Plants can offer a reliable alternative to conventional model systems to elucidate the concept of ENM biotransformation within tissues and organs, as a crucial step in understanding the mechanisms of ENM-organism interaction. To facilitate the understanding of the physico-chemical forms involved in plant response, synchrotron-based techniques have added new potential perspectives in studying the interactions between ENMs and biota. These techniques are providing new insights on the interactions between ENMs and biomolecules. The present review discusses the principal outcomes for ENMs after intake by plants, including possible routes of biotransformation which make their final

fate less uncertain, and therefore require further investigation.”

The Paragraph 3 has been also modified to give a more timely reference to potential applicability:

Lines 558-567: “This information is highly relevant with regard to potential applicability: ENMs can interact with sensitive ecosystem components within trophic food chains, affect microbial populations in soil, enter into the plant and where they can be translocated to different tissues and organs, including the edible tissues or organs (Holden et al., 2013; Liu et al., 2015). Biotransformation can occur at each step within these processes, modifying and/or amplifying ENM effects at organism level. These interactions from the level of ecosystem, organism, tissue, cell, and organelles become key factors when applying “ENM biotransformation” as a concept for a safer design, when considering applications for agriculture and food production, and for minimizing the adverse biological impact (Burello & Worth, 2015; Pagano et al., 2018; Lowry et al., 2019; Kah et al., 2019; Zulfiqar et al., 2019; Ma et al., 2020; Hameed et al., 2022; Xu et al., 2022).”

A new reference has been also included:

Xu T., Wang Y., Aytac Z., Zuverza-Mena N., Zhao Z., Hu X., Ng K.W., White J.C., Demokritou P.  
Enhancing Agrichemical Delivery and Plant Development with Biopolymer-Based Stimuli Responsive Core–Shell Nanostructures. *ACS Nano* 2022, 16, 4, 6034–6048.

You mentioned that three billion tons of crops are produced per year and cost a lot of source and energy in L41-47. What is the purpose? Please consider deleting them.

The new paragraph 1.1 entitled “from ENM exposure to biotransformation” has been reduced and thoroughly edited in order to be more focused on the main topic of the review:

Lines 47-99: “Although global food production has generally increased over time, the distribution has been far from equitable, with more than 820 million people having insufficient food and many more consuming low-quality diets leading directly to micronutrient deficiencies (Willett et al 2019; Zhong et al., 2020). In the past 20 years, and particularly in the past decade, engineered nanomaterials (ENMs) have seen dramatically increasing use and had an equally significant impact on ecosystems services and human society. As such, studies focused on the implications associated with their use are critical. In fact, it is known that ENMs exert important, but not completely understood, effects on biota; a particular topic of concern include the effect on crops, food production, and trophic transfer (Gardea-Torresdey et al., 2014; Ma et al., 2018; White et al., 2022).

The interplay between plant growth, dissolution, evaporation, and aggregation are key aspects of the dynamic behavior of ENMs in the environment. Directional aggregation can result in the formation of larger particles with a more complex morphology. However, given the complexity of natural environments, most nanomaterials can be found in hetero-aggregated composites of different inorganic and organic materials (Judy et al., 2012; Ma et al 2018). These aggregates can be very different from original simple pristine morphologies and may even form highly branched structures similar to fractals, all of which subsequently dramatically affect their reactivity and transport (Ma et al., 2018; Huangfu et al., 2019).

Sectors with a large nanomaterial application such as medicine and food production may experience greater risks of ENMs exposure due to their uses, with thousands of tons of ENMs that are eventually discarded into the three main environmental matrices: soil, water, and air (Keller et al., 2013; Zuverza Mena et al., 2017). ENMs with the greatest historical use include nanoscale ceria (nCeO<sub>2</sub>), silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva, 2014; Mitrano et al., 2015). Considering the long-time span of use of select materials (1950 to 2050), efforts to estimate ENMs release through commercial and associated activity into the environment have been undertaken. For example, for nSiO<sub>2</sub>, a global production between 100,000 and three million tons per year has been estimated, while

for nCeO<sub>2</sub>, levels likely reach the upper limit of 10,000 tons per year, and for nAg, the literature reflects a production volume below 1,000 tons per year (Giese et al., 2018). The use of nTiO<sub>2</sub> for the inhibition of microbial proliferation in food is one of the most important ways to prolong the shelf life of packaged products (Abutalib & Rajeh., 2020). However, a panel from EFSA concluded that E171 (TiO<sub>2</sub>) can no longer be considered as safe when used as a food additive (EFSA Journal 2021). Gottschalk et al., (2009) calculated environmental concentrations using a probabilistic LCA (life cycle analysis) of ENM containing products. The authors modelled nTiO<sub>2</sub>, nZnO, nAg, carbon nanotubes (CNT), and fullerenes for the U.S., Europe, and Switzerland. The concentrations in the environment were calculated through probabilistic density functions and compared to ecotoxicology data. In the simulations, the values ranged from 0.003 ng L<sup>-1</sup> (fullerenes) to 21 ng L<sup>-1</sup> (nTiO<sub>2</sub>) for surface waters and from 4 ng L<sup>-1</sup> for fullerenes to 4 µg L<sup>-1</sup> for nTiO<sub>2</sub> for sewage treatment effluents. In Europe and the U.S., ENMs increased annually in sludge treated soil, and ranged from 1 ng kg<sup>-1</sup> for fullerenes to 89 µg kg<sup>-1</sup> for nTiO<sub>2</sub> (Gottschalk et al. 2009; Keller et al., 2013; Keller & Lazareva, 2014; Rincon, 2019). Importantly, quantum dots (QDs), as well as many carbon- and metal-based ENMs, have been shown to produce negative effects on animals and plants as a function of dose, including accumulation, alteration of physiological and biochemical parameters, and reduced growth or yield (Oh et al., 2016; Zuverza-Mena et al., 2017). Quantum dots have shown to enter plant roots and to damage the cell wall, dysregulating metabolism (Marmioli et al., 2020). While mercaptoacetic acid (MAA)-coated CdSe/ZnS QDs induced minimal toxicity on maize seedlings, pristine cadmium/tellurium (Cd/Te) QDs induced chromatin stress, mitochondrial damage and inhibition on green gram sprouts (*Phaseolus radiatus* L.) growth (Song et al., 2013). There are several routes by which ENM can enter the soil-plant system. These include agricultural application of sewage sludges which often contain nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, and nAg; as well as the application of nano-enabled agrochemicals, resulting in the direct entry of nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, nFeOx, nCuO, CeO<sub>2</sub> and nAg into agricultural soils (Lv et al., 2019; Verma et al., 2022)."

Additional references included:

Gardea-Torresdey J.L., Rico C.M., White J.C. Trophic transfer, transformation, and impact of engineered nanomaterials in terrestrial environments. *Environ. Sci. Technol.* 2014, 48, 2526–2540.

Keller A.A., McFerran S., Lazareva A., Suh S. 2013. Global life cycle releases of engineered nanomaterials. *J. Nanopart. Res.* 15, 1692–1709.

Keller A.A., Lazareva A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environ. Sci. Technol. Lett.* 2014, 1, 1, 65–70.

Mitrano D.M., Motellier S., Clavaguera S., Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products, *Environment International*, 2015, 77, 132-147.

Rincon A.M. 2019. Chapter 6 - Presence of nanomaterials on consumer products: food, cosmetics, and drugs. pp. 165-181. Marmioli N., White J.C., Song J., Eds: In *Micro and Nano Technologies, Exposure to Engineered Nanomaterials in the Environment*, Elsevier, Amsterdam, The Netherlands.

Song U., Jun H., Waldman B., Roh J., Kim Y., Yi J., Lee E.J. Functional analyses of nanoparticle toxicity: a comparative study of the effects of TiO<sub>2</sub> and Ag on tomatoes (*Lycopersicon esculentum*) *Ecotoxicol. Environ. Saf.*, 2013. 93, 60-67.

White J.C., Zuverza-Mena N., Elmer W.H. From nanotoxicology to nano-enabled agriculture: Following the science at the Connecticut Agricultural Experiment Station (CAES). *Plant Nano Biology*, 2022, 1, 100007. Doi: 10.1016/j.plana.2022.100007.

L50-55 Please focus on plants, especially agricultural crops.

We agree with Rev1 give more relevance to the ENMs in agricultural practices. The sentence has been modified and two new references have been introduced.

Lines 52-55: "In fact, it is known that ENMs exert important, but not completely understood, effects on biota; a particular topic of concern include the effect on crops, food production, and trophic transfer (Gardea-Torresdey et al., 2014; Ma et al., 2018; White et al., 2022)."

References added:

Gardea-Torresdey J.L., Rico C.M., White J.C. Trophic transfer, transformation, and impact of engineered nanomaterials in terrestrial environments. *Environ. Sci. Technol.* 2014, 48, 2526–2540.

White J.C., Zuverza-Mena N., Elmer W.H. From nanotoxicology to nano-enabled agriculture: Following the science at the Connecticut Agricultural Experiment Station (CAES). *Plant Nano Biology*, 2022, 1, 100007. Doi: 10.1016/j.plana.2022.100007.

L53-54 It is too broad to conclude that the impacts of nanomaterials on biota are poorly understood. There are hundreds of publications investigating this topic.

The sentence, as suggested by Rev1, have been modified in order to be more topic oriented.

Lines 52-55: "In fact, it is known that ENMs exert important, but not completely understood, effects on biota; a particular topic of concern include the effect on crops, food production, and trophic transfer (Gardea-Torresdey et al., 2014; Ma et al., 2018; White et al., 2022)."

L67-68 Please add nCuO, nTiO<sub>2</sub>, and nZnO. nTiO<sub>2</sub> is the most used one among all the others.

Please check

[Ref]

[Ref]

The sentence has been modified as requested.

Lines 67-70: "ENMs with the greatest historical use include nanoscale ceria (nCeO<sub>2</sub>), silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva, 2014; Mitrano et al., 2015)."

The references suggested have been also added:

Keller A.A., Lazareva A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environ. Sci. Technol. Lett.* 2014, 1, 1, 65–70.

Mitrano D.M., Motellier S., Clavaguera S., Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products, *Environment International*, 2015, 77, 132-147.

L77 Can you find any latest references? 2009 is more than ten years ago.

Unfortunately, we have not found any new significant update on permissible concentrations, especially for the European side. From this point of view the cited paper is still maintained as an EU standards. Thus, we prefer to maintain this reference. To provide additional perspective, we included two additional references:

Keller A.A., McFerran S., Lazareva A., Suh S. 2013. Global life cycle releases of engineered nanomaterials. *J. Nanopart. Res.* 15, 1692–1709.

Keller A.A., Lazareva A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environ. Sci. Technol. Lett.* 2014, 1, 1, 65–70.

L92 Please refer to the right article.

We thank the Rev1 for the comment, the reference has been properly corrected and added to the references.

Song U., Jun H., Waldman B., Roh J., Kim Y., Yi J., Lee E.J. Functional analyses of nanoparticle toxicity: a comparative study of the effects of TiO<sub>2</sub> and Ag on tomatoes (*Lycopersicon esculentum*)  
*Ecotoxicol. Environ. Saf.*, 2013. 93, 60-67.

L99 In 1.1, "Once accumulated by plants" You haven't given any background regarding the uptake of ENMs by plants. At least add a few sentences to describe the possibility and give a few examples.

The beginning of the Paragraph, now 1.2, has been edited in order to give more information about the phases before plant accumulation. A new Figure 1 has been also included.

Lines 102-110: "Environmental and soil physico-chemical characteristics may significantly impact on ENMs aggregation, and dissolution, which may modify ENM bioavailability, uptake, translocation, and accumulation into terrestrial plants. In fact, light and temperature may induce potential changes on the ENM structure, such as during foliar spray (Hong et al., 2021). Once within plant tissues, ENM biotransformation may alter particle stability and behaviour in terms of interactions with biomolecules, triggering differential plant defense mechanisms (Ma et al., 2018; Rawat et al., 2018). ENMs are subject to a range of processes that may lead to their partial dissolution or result in structural modifications (Milosevic et al., 2020; Marmiroli et al., 2020). A schematic representation is reported in Figure 1."

New reference added:

Hong J., Wang C., Wagner D.C., Gardea-Torresdey J.L., He F., Rico C.M. 2021. Foliar application of nanoparticles: mechanisms of absorption, transfer, and multiple impacts. *Environ. Sci.: Nano*, 8, 1196-1210.

Rawat S., Pullagurula V.L.R., Adisa I.O., Wang Y., Peralta-Videa J.R., Gardea-Torresdey J.L. Factors affecting fate and transport of engineered nanomaterials in terrestrial environments, *COESH*, 2018, 6, 47-53.

L105-107 "biotransformation of nanomaterials lead to the attachment of biological molecules", or does the attachment of biological molecules lead to NPs biotransformation? Or both? Any examples?

The paragraph has been modified in order to avoid misinterpretation. A new Figure 1 has been also introduced.

Lines 110-129: "Nanoparticle biotransformation is a highly complex and poorly understood series of events and has been shown to occur during weathering in the soil, trophic transfer, and translocation within plant tissues. These reactions are highly dynamic and alter the original pristine structure of the nanoparticles in a number of ways, potentially causing the release of ions, but also the consequent restructuring (or destructuring) of the nanoparticle (Servin et al., 2017a). Biotransformation of nanomaterials may rest on the interaction with biological molecules that stabilize their external reactivity, such as peptides including those involved in detoxification, (e.g. glutathione), fatty acids, secondary metabolites, and even components of cell

membranes (Marmioli et al., 2020). Particle properties such as size, stability, charge, and dissolution may strongly influence other biotransformation mechanisms, potentially promoting enzymatic modification and functionalization with proteins (e.g., corona protein) present in the cytoplasm and organelles (Ma et al., 2018; Marmioli et al., 2020). ENMs may maintain crystal structure when internalized by cells or may be disassembled and converted into less complex structures (by biological modification or chelation), thus reducing toxicity, and the risk of their accumulation and translocation (Wang et al., 2022). These post-uptake structural modifications involve specific parameters such as bond distance with other atoms or nature of the ligand atoms. In consideration of this, one objective in biotransformation studies is to investigate the physico-chemical forms (e.g., nanocrystal structure) within exposed tissues and to characterize the structural differences within the new biotransformed molecules, including identification of the biomolecules interacting with the ENMs (Castillo-Michel et al., 2017; Marmioli et al., 2020).”

L110 More citations are needed to support the statement of "properties such as size, stability, charge, and dissolution influence the biotransformation". Besides, plant cultivars can also affect ENMs in planta biodistribution  
[Ref]

The paragraph has been modified in order to include new references and also the effect of the plant cultivars.

Lines 115-131: “Biotransformation of nanomaterials may rest on the interaction with biological molecules that stabilize their external reactivity, such as peptides including those involved in detoxification, (e.g. glutathione), fatty acids, secondary metabolites, and even components of cell membranes (Marmioli et al., 2020). Particle properties such as size, stability, charge, and dissolution may strongly influence other biotransformation mechanisms, potentially promoting enzymatic modification and functionalization with proteins (e.g., corona protein) present in the cytoplasm and organelles (Ma et al., 2018; Marmioli et al., 2020). ENMs may maintain crystal structure when internalized by cells or may be disassembled and converted into less complex structures (by biological modification or chelation), thus reducing toxicity, and the risk of their accumulation and translocation (Wang et al., 2022). These post-uptake structural modifications involve specific parameters such as bond distance with other atoms or nature of the ligand atoms. In consideration of this, one objective in biotransformation studies is to investigate the physico-chemical forms (e.g., nanocrystal structure) within exposed tissues and to characterize the structural differences within the new biotransformed molecules, including identification of the biomolecules interacting with the ENMs (Castillo-Michel et al., 2017; Marmioli et al., 2020). It has furthermore to consider how genetic diversity across different plant species and within the same plant species (in different cultivars) may influence the ENM uptake and translocation (Deng et al., 2020).”

New references included:

Deng C., Wang Y., Cota-Ruiz K., Reyes A., Sun Y., Peralta-Videa J.R., Hernandez-Viezcas J.A., Turley R.S., Niu G., Li C., Gardea-Torresdey J.L. Bok choy (Brassica rapa) grown in copper oxide nanoparticles-amended soils exhibits toxicity in a phenotype-dependent manner: Translocation, biodistribution and nutritional disturbance. *J Haz Mater*, 2020, 398, 122978.

Mitrano D.M., Motellier S., Clavaguera S., Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products, *Environment International*, 2015, 77, 132-147.

Rawat S., Pullagurala V.L.R., Adisa I.O., Wang Y., Peralta-Videa J.R., Gardea-Torresdey J.L. Factors affecting fate and transport of engineered nanomaterials in terrestrial environments, *COESH*, 2018, 6, 47-53.

Xu T., Wang Y., Aytac Z., Zuverza-Mena N., Zhao Z., Hu X., Ng K.W., White J.C., Demokritou P.



Enhancing Agrichemical Delivery and Plant Development with Biopolymer-Based Stimuli Responsive Core–Shell Nanostructures. ACS Nano 2022, 16, 4, 6034–6048.

L125-147 Please add a few sentences to compare the differences between these synchrotron-based techniques and non-synchrotron-supported ones, For example, u-XRF vs. TEM-EDS. What are the advantages?

According to the Abbe's Law the resolution power of a particle is d.

$d = \lambda / (2n \sin \alpha)$  In optical microscopy we can approximate d with  $\lambda$ .

In electron microscopy like TEM or SEM we use electrons that are accelerated passing through a potential difference they become thus equivalent to electromagnetic wave, as reported in the scheme.

Therefore, thanks to the De Broglie law we have  $\lambda(\text{nm}) = 1.22 / \sqrt{V}$ , where V is the acceleration voltage of the electrons.

In TEM V= around 30 to 100, thus  $\lambda$  varies between 0.007 nm and 0.004 nm respectively. The smallest of these resolutions are enough to see the electron lattice. With the synchrotron we use photon that do not have mass, therefore the factor 1.22 is substituted which is the non-relativistic mass of the electron which is  $m = 5.48579909065(16) \times 10^{-4}$  Da (according to The NIST Reference on Constants, Units, and Uncertainty. NIST. 20 May 2019. Retrieved 2020-06-21), this makes the resolution even smaller and increases the penetration depth into the sample.

The EDX is another thing because it depends on the acceleration voltage of the particle or of the photon. Every Element in the periodic table has its own orbitals energies, and the acceleration voltage allows to excite one or more of these, independently if it comes from a TEM or from a synchrotron. Hence in this respect the two techniques are alike. (Goldstein, Newbury et al Editors. Scanning Electron Microscopy and X-Ray Microanalysis, Third Edition. 2003 Springer Science+Business Media LLC, 233 Spring Street, New York, NY 10013 USA).

The text below has been added as a shortened version of these concepts.

Lines 160-165: "Synchrotron-based techniques use photons, which do not have mass; therefore the factor 1.22 is substituted by the non-relativistic mass of the electron which is  $m = 5.485 \times 10^{-4}$  Da. This makes the resolution even smaller and increases penetration depth into the sample. On the other hand, EDX depends on the acceleration voltage of the particle or of the photon. Every element has its own orbital energies, and the acceleration voltage allows excitation one or more of these, independently if it comes from a TEM or from a synchrotron (Goldstein et al., 2003)."

A new reference has been included:

Goldstein J.I., Newbury D.E., Echlin P., Joy D.C., Lyman C.E., Lifshin E., Sawyer L., Michael J.R. Editors. Scanning Electron Microscopy and X-Ray Microanalysis, Third Edition. 2003 Springer Science+Business Media LLC, 233 Spring Street, New York, NY 10013 USA.

L195 Add nano delivery system for smart release  
[Ref]

We thank Rev1 for the suggestion. The reference has been included and the text has been edited accordingly:

Lines 210-215: "Beyond the potential adverse effects upon bioaccumulation from soil or other exposure routes, there is an increasing interest in exploiting the potential positive effects of ENMs on plants, aiming to improve crop yields and quality. A range of mechanisms, including direct use as nanofertilizers (Verma et al., 2022), nanocarriers (Karny et al., 2018), smart delivery systems (Xu et al., 2022) or when in

association to plant growth-promoting bacteria, are considered (Prado de Moraes et al., 2021).”

Xu T., Wang Y., Aytac Z., Zuverza-Mena N., Zhao Z., Hu X., Ng K.W., White J.C., Demokritou P.  
Enhancing Agrichemical Delivery and Plant Development with Biopolymer-Based Stimuli Responsive Core–Shell Nanostructures. *ACS Nano* 2022, 16, 4, 6034–6048.

L251-267 Only one sentence was describing the synchrotron-based analysis. All the others were biomass or plant physiological responses. What does "limited stability of nLa2O3" mean? What are the results of the u-XRF analysis? Why is it unique compared to the other related analysis?

We thank Rev1 for the comment. The sentence has been clarified.

Lines 272-275:“The limited stability of nLa2O3, as compared to nCeO2, has been confirmed by -XRF analysis in *Cucumis sativus* L. through element speciation, dissolution studies in aqueous solution and in planta. After 14d treatment, the nCeO2 structure in the roots remains mostly preserved (more than 80%) while pristine nLa2O3 structure was observed at levels below 10% (Ma et al., 2015).”

Considering how Ce and La elemental properties play a fundamental role in their biotransformation, but also how their similarity in term of effects when analyzing the respective bulk forms, we believe that the direct comparison of the two nanoforms by XRF, as supported by data published in literature, could be an aspect to be described, and an interesting explanation for the physiological and molecular outcomes. Information about comparisons with other nanoforms has been reported at level of physiological and molecular analyses, both in terms of individual treatments, but also as a function of binary co-exposure (see Pagano et al., 2016, Pagano et al. 2017), which are reported in the text. This have been done with the aim to combine the potential results from physical and chemical analyses with results from physiological and molecular evidence, thereby providing a more mechanistic overview of the biotransformation processes.

L269 Please add more recently works:

[Ref]

[Ref]weathering effect in soil

The references indicated have been included and discussed.

Lines 312-318: “Interestingly, when either considering the utilization of pristine and coated nTiO2 (hydrophilic or hydrophobic) in carrot (*Daucus carota* L.), responses observed depended mainly on the nTiO2 surface coating, concentration and in soil weathering (Wang et al., 2021a; 2021b). Taproot and leaf fresh biomass and plant height were all increased with exposure, as well as nutrient uptake (Fe in leaves; Mg in taproots; Ca, Zn, K in roots). Conversely, sugar and starch contents were negatively affected, compromising the nutritional quality (Wang et al., 2021b).”

References included:

Wang Y., Deng C., Cota-Ruiz K., Tan W., Reyes A., Peralta-Videa J.R., Hernandez-Viezcas J.A., Li C., Gardea-Torresdey J.L. Effects of different surface-coated nTiO2 on full-grown carrot plants: Impacts on root splitting, essential elements, and Ti uptake. *J Haz Mater*, 2021a, 402, 123768.

Wang Y., Deng C., Cota-Ruiz K., Peralta-Videa J.R., Hernandez-Viezcas J.A., Gardea-Torresdey J.L. Soil-aged nano titanium dioxide effects on full-grown carrot: Dose and surface-coating dependent improvements on growth and nutrient quality. *Scie Tot Environ*, 2021b, 774, 145699.

L395-398 Please add more recent works regarding the nCuO-plant interactions.

Please check:

[Ref]

[Ref](in planta biodistribution of nCuO in the particle form and growth related gene expression)

The references indicated have been included and discussed.

Lines 452-464: "The grain of weedy and cultivated rice were differentially impacted by nCuO, bulk or ionic forms, showing also a cultivar-specific and concentration-dependent response. Cu translocation directly influenced plant yield, sugar production, starch content, protein content, and expression of auxin associated genes in grain (Deng et al., 2022b). Analyzing the effect of citric acid (CA) coated copper oxide (CA-nCuO) and its application (foliar spray or soil exposure) on the growth and physiology of soybean (*Glycine max* L.), nCuO appeared to be more accessible for plant uptake, as compared to CA-nCuO, decreasing the protein content, and inhibiting plant growth. CA reduced CuO NPs toxicity, demonstrating that surface modification may change the toxic properties of NPs (Deng et al., 2022c).

Treatment of *Lactuca sativa* L. with nCuO significantly increased biomass as compared to CuO microparticles (Wang et al., 2019). In addition, plants can benefit from nCuO treatment through enhanced defensive pathways, and through direct antimicrobial and antifungal activities (Elmer et al., 2018)."

References included:

Deng C., Wang Y., Navarro G., Sun Y., Cota-Ruiz K., Hernandez-Viezcas J.A., Niu G., Li C., White J.C., Gardea-Torresdey J. 2022b. Copper oxide (CuO) nanoparticles affect yield, nutritional quality, and auxin associated gene expression in weedy and cultivated rice (*Oryza sativa* L.) grains, *Scie Tot Environ*, 810, 152260.

Deng C., Wang Y., Cantu J.M., Valdes C., Navarro G., Cota-Ruiz K., Hernandez-Viezcas J.A., Li C., Elmer W.H., Dimkpa C.O., White J.C., Gardea-Torresdey, J.L. 2022c. Soil and foliar exposure of soybean (*Glycine max*) to Cu: Nanoparticle coating-dependent plant responses, *NanoImpact*, 26,100406.

Reviewer #2:

1. Please rewrite highlights 1 and 3. The highlight should point out the most critical findings, conclusions, or perspectives in this review.

Highlights have been modified in order to be more representative of the review text:

- Biotransformation is a fundamental phenomenon for understanding ENM-organism response mechanisms
- Synchrotron-based methodological analyses are critical for investigating ENM biotransformation
- Biotransformation of ENMs may have positive or negative effects when considering the agri-food application

2. Half of the text in this review are talking about the synchrotron-based analyses of ENM treated plant samples. However, it was not mentioned in the title, and the abstract only mentioned it once of its "increased use".

We thank Rev2 for the suggestions. Synchrotron-based techniques are becoming more important to comprehend the real physico-chemical forms of ENMs within plants tissues and organs and can give the missing information that we do not have from the physiological and molecular analyses, as suggested by the relevance of some of the most influential publications on this topic cited in the text (Castillo et al., 2017; Hameed et al., 2022). However, synchrotron-based techniques are not the main focus of the review. The mechanism of biotransformation behind the plant response to ENMs is the fundamental point. Synchrotron based techniques are certainly instrumental for shedding light on those mechanisms and the combination of results from physical analyses with physiological and molecular observations can give a more

comprehensive picture of what happens during ENMs treatment in planta. We are aware of the relevance of Synchrotron based techniques and we decided to give them an appropriate space in the introduction, in the (new) paragraph 1.2.

For these reasons we believe that title represents our work in the proper manner, and we would like to maintain it in the present form, where the emphasis is more on the mechanisms, while the techniques are tools to clarify those mechanisms.

The abstract, on the other hand, has been modified on order to be more informative on the points discussed in the main text:

“Research on engineered nanomaterials (ENMs) exposure has continued to expand rapidly, with a focus on uncovering the underlying mechanisms. The EU largely limits the number and the type of organisms that can be used for experimental testing through the 3R normative. There are different routes through which ENMs can enter the soil-plant system: this includes the agricultural application of sewage sludges, and the distribution of nano-enabled agrochemicals. However, a thorough understanding of the physiological and molecular implications of ENMs dispersion and chronic low-dose exposure remains elusive, thus requiring new evidence and a more mechanistic overview of pathways and major effectors involved in plants. Plants can offer a reliable alternative to conventional model systems to elucidate the concept of ENM biotransformation within tissues and organs, as a crucial step in understanding the mechanisms of ENM-organism interaction. To facilitate the understanding of the physico-chemical forms involved in plant response, synchrotron-based techniques have added new potential perspectives in studying the interactions between ENMs and biota. These techniques are providing new insights on the interactions between ENMs and biomolecules. The present review discusses the principal outcomes for ENMs after intake by plants, including possible routes of biotransformation which make their final fate less uncertain, and therefore require further investigation.”

3. The whole introduction (from L41-L96) is talking about the reported environmental exposure of ENM and the effects of quantum dots on plant growth. Please revise this part and be more focused on your main topic. This review aims to summarize the synchrotron-based analysis of the ENM biotransformation in plants and some molecular effects.

We thank Rev2 for the comment. Introduction has been modified in order to be more topic oriented.

Lines 47-99: “Although global food production has generally increased over time, the distribution has been far from equitable, with more than 820 million people having insufficient food and many more consuming low-quality diets leading directly to micronutrient deficiencies (Willett et al 2019; Zhong et al., 2020). In the past 20 years, and particularly in the past decade, engineered nanomaterials (ENMs) have seen dramatically increasing use and had an equally significant impact on ecosystems services and human society. As such, studies focused on the implications associated with their use are critical. In fact, it is known that ENMs exert important, but not completely understood, effects on biota; a particular topic of concern include the effect on crops, food production, and trophic transfer (Gardea-Torresdey et al., 2014; Ma et al., 2018; White et al., 2022).

The interplay between plant growth, dissolution, evaporation, and aggregation are key aspects of the dynamic behavior of ENMs in the environment. Directional aggregation can result in the formation of larger particles with a more complex morphology. However, given the complexity of natural environments, most nanomaterials can be found in hetero-aggregated composites of different inorganic and organic materials (Judy et al., 2012; Ma et al 2018). These aggregates can be very different from original simple pristine morphologies and may even form highly branched structures similar to fractals, all of which subsequently dramatically affect their reactivity and transport (Ma et al., 2018; Huangfu et al., 2019).

Sectors with a large nanomaterial application such as medicine and food production may experience greater risks of ENMs exposure due to their uses, with thousands of tons of ENMs that are eventually discarded into the three main environmental matrices: soil, water, and air (Keller et al., 2013; Zuverza Mena et al., 2017). ENMs with the

greatest historical use include nanoscale ceria (nCeO<sub>2</sub>), silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva, 2014; Mitrano et al., 2015). Considering the long-time span of use of select materials (1950 to 2050), efforts to estimate ENMs release through commercial and associated activity into the environment have been undertaken. For example, for nSiO<sub>2</sub>, a global production between 100,000 and three million tons per year has been estimated, while for nCeO<sub>2</sub>, levels likely reach the upper limit of 10,000 tons per year, and for nAg, the literature reflects a production volume below 1,000 tons per year (Giese et al., 2018). The use of nTiO<sub>2</sub> for the inhibition of microbial proliferation in food is one of the most important ways to prolong the shelf life of packaged products (Abutalib & Rajeh., 2020). However, a panel from EFSA concluded that E171 (TiO<sub>2</sub>) can no longer be considered as safe when used as a food additive (EFSA Journal 2021). Gottschalk et al., (2009) calculated environmental concentrations using a probabilistic LCA (life cycle analysis) of ENM containing products. The authors modelled nTiO<sub>2</sub>, nZnO, nAg, carbon nanotubes (CNT), and fullerenes for the U.S., Europe, and Switzerland. The concentrations in the environment were calculated through probabilistic density functions and compared to ecotoxicology data. In the simulations, the values ranged from 0.003 ng L<sup>-1</sup> (fullerenes) to 21 ng L<sup>-1</sup> (nTiO<sub>2</sub>) for surface waters and from 4 ng L<sup>-1</sup> for fullerenes to 4 µg L<sup>-1</sup> for nTiO<sub>2</sub> for sewage treatment effluents. In Europe and the U.S., ENMs increased annually in sludge treated soil, and ranged from 1 ng kg<sup>-1</sup> for fullerenes to 89 µg kg<sup>-1</sup> for nTiO<sub>2</sub> (Gottschalk et al. 2009; Keller et al., 2013; Keller & Lazareva, 2014; Rincon, 2019). Importantly, quantum dots (QDs), as well as many carbon- and metal-based ENMs, have been shown to produce negative effects on animals and plants as a function of dose, including accumulation, alteration of physiological and biochemical parameters, and reduced growth or yield (Oh et al., 2016; Zuverza-Mena et al., 2017). Quantum dots have shown to enter plant roots and to damage the cell wall, dysregulating metabolism (Marmioli et al., 2020). While mercaptoacetic acid (MAA)-coated CdSe/ZnS QDs induced minimal toxicity on maize seedlings, pristine cadmium/tellurium (Cd/Te) QDs induced chromatin stress, mitochondrial damage and inhibition on green gram sprouts (*Phaseolus radiatus* L.) growth (Song et al., 2013). There are several routes by which ENM can enter the soil-plant system. These include agricultural application of sewage sludges which often contain nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, and nAg; as well as the application of nano-enabled agrochemicals, resulting in the direct entry of nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, nFeOx, nCuO, CeO<sub>2</sub> and nAg into agricultural soils (Lv et al., 2019; Verma et al., 2022).”

Additional references included:

Gardea-Torresdey J.L., Rico C.M., White J.C. Trophic transfer, transformation, and impact of engineered nanomaterials in terrestrial environments. *Environ. Sci. Technol.* 2014, 48, 2526–2540.

Keller A.A., McFerran S., Lazareva A., Suh S. 2013. Global life cycle releases of engineered nanomaterials. *J. Nanopart. Res.* 15, 1692–1709.

Keller A.A., Lazareva A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environ. Sci. Technol. Lett.* 2014, 1, 1, 65–70.

Mitrano D.M., Motellier S., Clavaguera S., Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products, *Environment International*, 2015, 77, 132-147.

Rincon A.M. 2019. Chapter 6 - Presence of nanomaterials on consumer products: food, cosmetics, and drugs. pp. 165-181. Marmioli N., White J.C., Song J., Eds: In *Micro and Nano Technologies, Exposure to Engineered Nanomaterials in the Environment*, Elsevier, Amsterdam, The Netherlands.

Song U., Jun H., Waldman B., Roh J., Kim Y., Yi J., Lee E.J. Functional analyses of nanoparticle toxicity: a comparative study of the effects of TiO<sub>2</sub> and Ag on tomatoes (*Lycopersicon esculentum*) *Ecotoxicol. Environ. Saf.*, 2013. 93, 60-67.

White J.C., Zuverza-Mena N., Elmer W.H. From nanotoxicology to nano-enabled agriculture: Following the science at the Connecticut Agricultural Experiment Station (CAES). *Plant Nano Biology*, 2022, 1, 100007. Doi: 10.1016/j.plana.2022.100007.

4. L66 Please add more appropriate citations. Can you provide any related numbers?

New references have been added in order to give more quantitative information:

Lines 64-70: "Sectors with a large nanomaterial application such as medicine and food production may experience greater risks of ENMs exposure due to their uses, with thousands of tons of ENMs that are eventually discarded into the three main environmental matrices: soil, water, and air (Keller et al., 2013; Zuverza Mena et al., 2017). ENMs with the greatest historical use include nanoscale ceria (nCeO<sub>2</sub>), silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva, 2014; Mitrano et al., 2015)."

New references included:

Keller A.A., McFerran S., Lazareva A., Suh S. 2013. Global life cycle releases of engineered nanomaterials. *J. Nanopart. Res.* 15, 1692–1709.

Keller A.A., Lazareva A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environ. Sci. Technol. Lett.* 2014, 1, 1, 65–70.

Mitrano D.M., Motellier S., Clavaguera S., Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products, *Environment International*, 2015, 77, 132-147.

5. L67 and L78 Please define nSiO<sub>2</sub>, nAg, nZnO, and so forth.

We thank Rev2 for the comment. We included the definition of each ENM cited:

Lines 67-70: "ENMs with the greatest historical use include nanoscale ceria (nCeO<sub>2</sub>), silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva, 2014; Mitrano et al., 2015)."

6. L71 Please add a citation.

The sentence has been fixed in order to give the proper citation.

Lines 72-75: "For example, for nSiO<sub>2</sub>, a global production between 100,000 and three million tons per year has been estimated, while for nCeO<sub>2</sub>, levels likely reach the upper limit of 10,000 tons per year, and for nAg, the literature reflects a production volume below 1,000 tons per year (Giese et al., 2018)."

7. L73 TiO<sub>2</sub> or nanoscale TiO<sub>2</sub>?

The typo has been fixed.

8. You only have one subsection "1.1" in part 1. Please consider merging it into part 1. Or change it to 1.2, and put all the content before it into 1.1.

Paragraph subdivision has been updated, including an initial subsection called "1. Engineered nanomaterial (ENM) biotransformation". The first section is now "1.1. ENMs: from exposure to biotransformation", while the previous section 1.1 is now shifted to 1.2.

9. L101-105 Please check

[Ref]

[Ref] for factors and mechanisms of the NPs biotransformation, especially the effect of soil weathering on plant responses.

We thank Rev2 for the suggestions. The text has been updated, as reported:

Lines 102-121: "Environmental and soil physico-chemical characteristics may significantly impact on ENMs aggregation, and dissolution, which may modify ENM bioavailability, uptake, translocation, and accumulation into terrestrial plants. In fact, light and temperature may induce potential changes on the ENM structure, such as during foliar spray (Hong et al., 2021). Once within plant tissues, ENM biotransformation may alter particle stability and behaviour in terms of interactions with biomolecules, triggering differential plant defense mechanisms (Ma et al., 2018; Rawat et al., 2018). ENMs are subject to a range of processes that may lead to their partial dissolution or result in structural modifications (Milosevic et al., 2020; Marmioli et al., 2020). A schematic representation is reported in Figure 1. Nanoparticle biotransformation is a highly complex and poorly understood series of events and has been shown to occur during weathering in the soil, trophic transfer, and translocation within plant tissues. These reactions are highly dynamic and alter the original pristine structure of the nanoparticles in a number of ways, potentially causing the release of ions, but also the consequent restructuring (or destructuring) of the nanoparticle (Servin et al., 2017a). Biotransformation of nanomaterials may rest on the interaction with biological molecules that stabilize their external reactivity, such as peptides including those involved in detoxification, (e.g. glutathione), fatty acids, secondary metabolites, and even components of cell membranes (Marmioli et al., 2020). Particle properties such as size, stability, charge, and dissolution may strongly influence other biotransformation mechanisms, potentially promoting enzymatic modification and functionalization with proteins (e.g., corona protein) present in the cytoplasm and organelles (Ma et al., 2018; Marmioli et al., 2020)."

A new reference has been included, as well as an additional Figure (new Figure 1):

Hong J., Wang C., Wagner D.C., Gardea-Torresdey J.L., He F., Rico C.M. 2021. Foliar application of nanoparticles: mechanisms of absorption, transfer, and multiple impacts. *Environ. Sci.: Nano*, 8, 1196-1210.

Rawat S., Pullagurala V.L.R., Adisa I.O., Wang Y., Peralta-Videa J.R., Gardea-Torresdey J.L. Factors affecting fate and transport of engineered nanomaterials in terrestrial environments, *COESH*, 2018, 6, 47-53.

The suggested reference related to "Soil-aged nano titanium dioxide effects on full-grown carrot" has been included in the main text in the case studies reported in the paragraph 2.2 as Wang et al., 2021b.

10. L110-113 Besides the listed possibilities, ENMs can also remain as nanoscale particles with/without the loss of surface coatings in plant tissues after being uptaken. Please check

[ref]

We thank Rev 2 for the suggestion. The reference has been cited, as reported.

Lines 121-124: "ENMs may maintain crystal structure when internalized by cells or may be disassembled and converted into less complex structures (by biological modification or chelation), thus reducing toxicity, and the risk of their accumulation and translocation (Wang et al., 2022)."

11. L178-181 Add more latest references. Please check

[ref]

[ref]

We thank Rev2 for the suggestions. The text has been updated, as reported:

Lines 196-199: "Studies have added significant molecular data on the effects of ENMs exposure in plants (Schwab et al., 2016; Ma et al., 2018). Different results are often observed for the same element as a function of its form or size, i.e. nanostructured, bulk, or ionic species (Pagano et al., 2016; Wang et al., 2022)."

The suggested reference related to "Copper oxide (CuO) nanoparticles affect yield, nutritional quality, and auxin associated gene expression in weedy and cultivated rice" has been included in the text in the case studies reported in the paragraph 2.6 as Deng et al., 2022b.

12. L202 Can you briefly describe Table 1? What is Table 1 for and why are genes listed? Were they significantly affected by the ENM exposure? All the citations listed in Table 1 are related to the author themselves. Please include some works from the others.

Genes reported in Figure 2 (former Figure 1) and Table 1 are some of those that are modulated by the different type of ENMs as reported in the cited studies. Other genes also resulted responsiveness in a ENM-specific manner during exposure and are considered for this reason as potential biomarker of exposure/effect. Moreover, these specific biomarkers are also able to testify how the modulation of the genes in the different organs is in some cases convergent between differential forms (nano, bulk, ion) of the same element. Considering that the concept of nano-specific biomarker has been introduced in some of our research groups papers, we thought to include these references in the Table 1. This was not the case of Table 2, in which papers not related to our research group on the principal evidence of the ENM biotransformation in plant observed by physiological, molecular and synchrotron-based analyses have been included.

Table 1 description has b...



To Co-Editors-in-Chief, Environmental Pollution Journal

Prof. Jörg Rinklebe,

Prof. Christian Sonne,

Prof. Eddy Zeng

To Special Issue Editor,

Prof. Da Chen

To Guest Editor,

Prof. M.H. Siddiqui

Special Issue “Emerging Pollutants and their effects on plants: present and future challenges, and their solutions”

Dear Editor,

We thank the Editor and Reviewers for their comments and suggestions which gave the opportunity to improve our Review manuscript “Nanomaterials biotransformation: In planta mechanisms of action” in all its aspects.

As requested, the manuscript has been modified, including the abstract, highlights and paragraph subdivisions (in the introduction). In addition, the introduction has been thoroughly edited. A list of new references has been included. A new Figure 1 has been included in order to give more explanations to the biotransformation mechanisms.

Below are the point-to-point answers to Reviewers queries.

We believe the manuscript has been strongly improved and hope it is now acceptable for publication.

Kind Regards

Marta Marmiroli

To Co-Editors-in-Chief, Environmental Pollution Journal

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Prof. Christian Sonne,  
Prof. Eddy Zeng

To Special Issue Editor,

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We believe the manuscript has been strongly improved and hope it is now acceptable for publication.

Kind Regards

Marta Marmiroli

*Editor and Reviewer comments:*

**Reviewer #1:** *Manuscript review of ENVPOL-D-22-05734 "Nanomaterials biotransformation: In planta mechanisms of action"*

*This review article summarized recent works on the nanomaterial biotransformation within plant tissues using synchrotron-based techniques. The impacts of engineered nanomaterials (ENMs) on plant gene expression were also included. Specifically, studies regarding the biointeraction between plants and ENMs (nanoscale CeO<sub>2</sub>, La<sub>2</sub>O<sub>3</sub>, TiO<sub>2</sub>, Au, FeO<sub>x</sub>, ZVI, ZnO, CuO, and CdS QDs) were listed and discussed in detail. This review paper contributes to the deep understanding of the fate of ENMs in plant tissues, and the related genetic regulation of plants induced by ENM exposures. The contents are within the scope of the Environmental Pollution. However, I have a number of general concerns, followed by a range of specific comments, which prevent me from recommending this paper for publication in its current form.*

*General Comments-*

*Some parts of the abstract and introduction are not quite relevant to the main topic, and need to be revised. The main topic, synchrotron-based analysis of ENMs biotransformation, should be more emphasized and discussed in more detail. In part 1, there was only one sub-title 1.1. None of the figures or the tables provided any information related to the in planta biotransformation. Several figures with summarized information instead of only one figure are better for a review article. Some references are too old or not representative. They should be up to date.*

We thank Rev1 for the constructive comments and suggestions given. The manuscript has been thoroughly improved in order to answer to all comments reported. Paragraph subdivision has been updated. Several new references and an additional Figure have been included. All the edited sections have been reported in the answer to each specific question.

*Specific Comments-*

*The abstract used a large amount of space describing exposure pathways and low-dose effects. However, they were not well discussed in the main text. Similarly, the "potential applicability" was not given in the main text. The abstract should contain the most significant findings, critical comments on the current studies, or perspectives for future research.*

The abstract has been modified in order to be more informative on the points discussed in the main text. The abstract now reads as follows:

“Research on engineered nanomaterials (ENMs) exposure has continued to expand rapidly, with a focus on uncovering the underlying mechanisms. The EU largely limits the number and the type of organisms that can be used for experimental testing through the 3R normative. There are different routes through which ENMs can enter the soil-plant system: this includes the agricultural application of sewage sludges, and the distribution of nano-enabled agrochemicals. However, a thorough understanding of the physiological and molecular implications of ENMs dispersion and chronic low-dose exposure remains elusive, thus requiring new evidence and a more mechanistic overview of pathways and major effectors involved in plants. Plants can offer a reliable alternative to conventional model systems to elucidate the concept of ENM biotransformation within tissues and organs, as a crucial step in understanding the mechanisms of ENM-organism interaction. To facilitate the understanding of the physico-chemical forms involved in plant response, synchrotron-based techniques have added new potential perspectives in studying the interactions between ENMs and

biota. These techniques are providing new insights on the interactions between ENMs and biomolecules. The present review discusses the principal outcomes for ENMs after intake by plants, including possible routes of biotransformation which make their final fate less uncertain, and therefore require further investigation.”

The Paragraph 3 has been also modified to give a more timely reference to potential applicability:

Lines 558-567: “This information is highly relevant with regard to potential applicability: ENMs can interact with sensitive ecosystem components within trophic food chains, affect microbial populations in soil, enter into the plant and where they can be translocated to different tissues and organs, including the edible tissues or organs (Holden et al., 2013; Liu et al., 2015). Biotransformation can occur at each step within these processes, modifying and/or amplifying ENM effects at organism level. These interactions from the level of ecosystem, organism, tissue, cell, and organelles become key factors when applying “ENM biotransformation” as a concept for a safer design, when considering applications for agriculture and food production, and for minimizing the adverse biological impact (Burello & Worth, 2015; Pagano et al., 2018; Lowry et al., 2019; Kah et al., 2019; Zulfiqar et al., 2019; Ma et al., 2020; Hameed et al., 2022; Xu et al., 2022).”

A new reference has been also included:

Xu T., Wang Y., Aytac Z., Zuverza-Mena N., Zhao Z., Hu X., Ng K.W., White J.C., Demokritou P. Enhancing Agrichemical Delivery and Plant Development with Biopolymer-Based Stimuli Responsive Core–Shell Nanostructures. *ACS Nano* 2022, 16, 4, 6034–6048.

*You mentioned that three billion tons of crops are produced per year and cost a lot of source and energy in L41-47. What is the purpose? Please consider deleting them.*

The new paragraph 1.1 entitled “from ENM exposure to biotransformation” has been reduced and thoroughly edited in order to be more focused on the main topic of the review:

Lines 47-99: “Although global food production has generally increased over time, the distribution has been far from equitable, with more than 820 million people having insufficient food and many more consuming low-quality diets leading directly to micronutrient deficiencies (Willett et al 2019; Zhong et al., 2020). In the past 20 years, and particularly in the past decade, engineered nanomaterials (ENMs) have seen dramatically increasing use and had an equally significant impact on ecosystems services and human society. As such, studies focused on the implications associated with their use are critical. In fact, it is known that ENMs exert important, but not completely understood, effects on biota; a particular topic of concern include the effect on crops, food production, and trophic transfer (Gardea-Torresdey et al., 2014; Ma et al., 2018; White et al., 2022).

The interplay between plant growth, dissolution, evaporation, and aggregation are key aspects of the dynamic behavior of ENMs in the environment. Directional aggregation can result in the formation of larger particles with a more complex morphology. However, given the complexity of natural environments, most nanomaterials can be found in hetero-aggregated composites of different inorganic and organic materials (Judy et al., 2012; Ma et al 2018). These aggregates can be very different from original simple pristine morphologies and may even form highly branched structures similar to fractals, all of which subsequently dramatically affect their reactivity and transport (Ma et al., 2018; Huangfu et al., 2019).

Sectors with a large nanomaterial application such as medicine and food production may experience greater risks of ENMs exposure due to their uses, with thousands of tons of ENMs that are eventually discarded into the three main environmental matrices: soil, water, and air (Keller et al., 2013; Zuverza Mena et al., 2017). ENMs with the greatest historical use include nanoscale ceria (nCeO<sub>2</sub>), silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva, 2014; Mitrano et al., 2015). Considering the long-time span of use of select materials (1950 to 2050), efforts to estimate ENMs release through commercial and associated activity into the environment have been undertaken. For example, for nSiO<sub>2</sub>, a global production between 100,000 and three million tons per year has been estimated, while for nCeO<sub>2</sub>, levels likely reach the upper limit of 10,000 tons per year, and for nAg, the literature reflects a production volume below 1,000 tons per year (Giese et al., 2018). The use of nTiO<sub>2</sub> for the inhibition of microbial proliferation in food is one of the most important ways to prolong the shelf life of packaged products (Abutalib & Rajeh., 2020). However, a panel from EFSA concluded that E171 (TiO<sub>2</sub>) can no longer be considered as safe when used as a food additive (EFSA Journal 2021).

Gottschalk et al., (2009) calculated environmental concentrations using a probabilistic LCA (life cycle analysis) of ENM containing products. The authors modelled nTiO<sub>2</sub>, nZnO, nAg, carbon nanotubes (CNT), and fullerenes for the U.S., Europe, and Switzerland. The concentrations in the environment were calculated through probabilistic density functions and compared to ecotoxicology data. In the simulations, the values ranged from 0.003 ng L<sup>-1</sup> (fullerenes) to 21 ng L<sup>-1</sup> (nTiO<sub>2</sub>) for surface waters and from 4 ng L<sup>-1</sup> for fullerenes to 4 µg L<sup>-1</sup> for nTiO<sub>2</sub> for sewage treatment effluents. In Europe and the U.S., ENMs increased annually in sludge treated soil, and ranged from 1 ng kg<sup>-1</sup> for fullerenes to 89 µg kg<sup>-1</sup> for nTiO<sub>2</sub> (Gottschalk et al. 2009; Keller et al., 2013; Keller & Lazareva, 2014; Rincon, 2019).

Importantly, quantum dots (QDs), as well as many carbon- and metal-based ENMs, have been shown to produce negative effects on animals and plants as a function of dose, including accumulation, alteration of physiological and biochemical parameters, and reduced growth or yield (Oh et al., 2016; Zuverza-Mena et al., 2017). Quantum dots have shown to enter plant roots and to damage the cell wall, dysregulating metabolism (Marmioli et al., 2020). While mercaptoacetic acid (MAA)-coated CdSe/ZnS QDs induced minimal toxicity on maize seedlings, pristine cadmium/tellurium (Cd/Te) QDs induced chromatin stress, mitochondrial damage and inhibition on green gram sprouts (*Phaseolus radiatus* L.) growth (Song et al., 2013). There are several routes by which ENM can enter the soil-plant system. These include agricultural application of sewage sludges which often contain nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, and nAg; as well as the application of nano-enabled agrochemicals, resulting in the direct entry of nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, nFeOx, nCuO, CeO<sub>2</sub> and nAg into agricultural soils (Lv et al., 2019; Verma et al., 2022).”

Additional references included:

Gardea-Torresdey J.L., Rico C.M., White J.C. Trophic transfer, transformation, and impact of engineered nanomaterials in terrestrial environments. *Environ. Sci. Technol.* 2014, 48, 2526–2540.

Keller A.A., McFerran S., Lazareva A., Suh S. 2013. Global life cycle releases of engineered nanomaterials. *J. Nanopart. Res.* 15, 1692–1709.

Keller A.A., Lazareva A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environ. Sci. Technol. Lett.* 2014, 1, 1, 65–70.

Mitrano D.M., Motellier S., Clavaguera S., Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products, *Environment International*, 2015,

77, 132-147.

Rincon A.M. 2019. Chapter 6 - Presence of nanomaterials on consumer products: food, cosmetics, and drugs. pp. 165-181. Marmiroli N., White J.C., Song J., Eds: In *Micro and Nano Technologies, Exposure to Engineered Nanomaterials in the Environment*, Elsevier, Amsterdam, The Netherlands.

Song U., Jun H., Waldman B., Roh J., Kim Y., Yi J., Lee E.J. Functional analyses of nanoparticle toxicity: a comparative study of the effects of TiO<sub>2</sub> and Ag on tomatoes (*Lycopersicon esculentum*) *Ecotoxicol. Environ. Saf.*, 2013. 93, 60-67.

White J.C., Zuverza-Mena N., Elmer W.H. From nanotoxicology to nano-enabled agriculture: Following the science at the Connecticut Agricultural Experiment Station (CAES). *Plant Nano Biology*, 2022, 1, 100007. Doi: 10.1016/j.plana.2022.100007.

*L50-55 Please focus on plants, especially agricultural crops.*

We agree with Rev1 give more relevance to the ENMs in agricultural practices. The sentence has been modified and two new references have been introduced.

Lines 52-55: “In fact, it is known that ENMs exert important, but not completely understood, effects on biota; a particular topic of concern include the effect on crops, food production, and trophic transfer (Gardea-Torresdey et al., 2014; Ma et al., 2018; White et al., 2022).”

References added:

Gardea-Torresdey J.L., Rico C.M., White J.C. Trophic transfer, transformation, and impact of engineered nanomaterials in terrestrial environments. *Environ. Sci. Technol.* 2014, 48, 2526–2540.

White J.C., Zuverza-Mena N., Elmer W.H. From nanotoxicology to nano-enabled agriculture: Following the science at the Connecticut Agricultural Experiment Station (CAES). *Plant Nano Biology*, 2022, 1, 100007. Doi: 10.1016/j.plana.2022.100007.

*L53-54 It is too broad to conclude that the impacts of nanomaterials on biota are poorly understood. There are hundreds of publications investigating this topic.*

The sentence, as suggested by Rev1, have been modified in order to be more topic oriented.

Lines 52-55: “In fact, it is known that ENMs exert important, but not completely understood, effects on biota; a particular topic of concern include the effect on crops, food production, and trophic transfer (Gardea-Torresdey et al., 2014; Ma et al., 2018; White et al., 2022).”

*L67-68 Please add nCuO, nTiO<sub>2</sub>, and nZnO. nTiO<sub>2</sub> is the most used one among all the others.*

*Please check*

*[Ref]*

*[Ref]*

The sentence has been modified as requested.

Lines 67-70: “ENMs with the greatest historical use include nanoscale ceria (nCeO<sub>2</sub>), silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva, 2014; Mitrano et al., 2015).”

The references suggested have been also added:

Keller A.A., Lazareva A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. Environ. Sci. Technol. Lett. 2014, 1, 1, 65–70.

Mitrano D.M., Motellier S., Clavaguera S., Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products, Environment International, 2015, 77, 132-147.

*L77 Can you find any latest references? 2009 is more than ten years ago.*

Unfortunately, we have not found any new significant update on permissible concentrations, especially for the European side. From this point of view the cited paper is still maintained as an EU standards. Thus, we prefer to maintain this reference. To provide additional perspective, we included two additional references:

Keller A.A., McFerran S., Lazareva A., Suh S. 2013. Global life cycle releases of engineered nanomaterials. J. Nanopart. Res. 15, 1692–1709.

Keller A.A., Lazareva A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. Environ. Sci. Technol. Lett. 2014, 1, 1, 65–70.

*L92 Please refer to the right article.*

We thank the Rev1 for the comment, the reference has been properly corrected and added to the references.

Song U., Jun H., Waldman B., Roh J., Kim Y., Yi J., Lee E.J. Functional analyses of nanoparticle toxicity: a comparative study of the effects of TiO<sub>2</sub> and Ag on tomatoes (*Lycopersicon esculentum*) Ecotoxicol. Environ. Saf., 2013. 93, 60-67.

*L99 In 1.1, "Once accumulated by plants" You haven't given any background regarding the uptake of ENMs by plants. At least add a few sentences to describe the possibility and give a few examples.*

The beginning of the Paragraph, now 1.2, has been edited in order to give more information about the phases before plant accumulation. A new Figure 1 has been also included.



Lines 102-110: “Environmental and soil physico-chemical characteristics may significantly impact on ENMs aggregation, and dissolution, which may modify ENM bioavailability, uptake, translocation, and accumulation into terrestrial plants. In fact, light and temperature may induce potential changes on the ENM structure, such as during foliar spray (Hong et al., 2021). Once within plant tissues, ENM biotransformation may alter particle stability and behaviour in terms of interactions with biomolecules, triggering differential plant defense mechanisms (Ma et al., 2018; Rawat et al., 2018). ENMs are subject to a range of processes that may lead to their partial dissolution or result in structural modifications (Milosevic et al., 2020; Marmiroli et al., 2020). A schematic representation is reported in Figure 1.”

New reference added:

Hong J., Wang C., Wagner D.C., Gardea-Torresdey J.L., He F., Rico C.M. 2021. Foliar application of nanoparticles: mechanisms of absorption, transfer, and multiple impacts. *Environ. Sci.: Nano*, 8, 1196-1210.

Rawat S., Pullagurala V.L.R., Adisa I.O., Wang Y., Peralta-Videa J.R., Gardea-Torresdey J.L. Factors affecting fate and transport of engineered nanomaterials in terrestrial environments, *COESH*, 2018, 6, 47-53.

*L105-107 "biotransformation of nanomaterials lead to the attachment of biological molecules", or does the attachment of biological molecules lead to NPs biotransformation? Or both? Any examples?*

The paragraph has been modified in order to avoid misinterpretation. A new Figure 1 has been also introduced.

Lines 110-129: “Nanoparticle biotransformation is a highly complex and poorly understood series of events and has been shown to occur during weathering in the soil, trophic transfer, and translocation within plant tissues. These reactions are highly dynamic and alter the original pristine structure of the nanoparticles in a number of ways, potentially causing the release of ions, but also the consequent restructuring (or destructuring) of the nanoparticle (Servin et al., 2017a). Biotransformation of nanomaterials may rest on the interaction with biological molecules that stabilize their external reactivity, such as peptides including those involved in detoxification, (*e.g.* glutathione), fatty acids, secondary metabolites, and even components of cell membranes (Marmiroli et al., 2020). Particle properties such as size, stability, charge, and dissolution may strongly influence other biotransformation mechanisms, potentially promoting enzymatic modification and functionalization with proteins (*e.g.*, corona protein) present in the cytoplasm and organelles (Ma et al., 2018; Marmiroli et al., 2020). ENMs may maintain crystal structure when internalized by cells or may be disassembled and converted into less complex structures (by biological modification or chelation), thus reducing toxicity, and the risk of their accumulation and translocation (Wang et al., 2022). These post-uptake structural modifications involve specific parameters such as bond distance with other atoms or nature of the ligand atoms. In consideration of this, one objective in biotransformation studies is to investigate the physico-chemical forms (*e.g.*, nanocrystal structure) within exposed tissues and to characterize the structural differences within the new biotransformed molecules, including identification of the biomolecules interacting with the ENMs (Castillo-Michel et al., 2017; Marmiroli et al., 2020).”

*L110 More citations are needed to support the statement of "properties such as size, stability, charge, and dissolution influence the biotransformation". Besides, plant cultivars can also affect ENMs in planta biodistribution*  
[Ref]

The paragraph has been modified in order to include new references and also the effect of the plant cultivars.

Lines 115-131: “Biotransformation of nanomaterials may rest on the interaction with biological molecules that stabilize their external reactivity, such as peptides including those involved in detoxification, (e.g. glutathione), fatty acids, secondary metabolites, and even components of cell membranes (Marmioli et al., 2020). Particle properties such as size, stability, charge, and dissolution may strongly influence other biotransformation mechanisms, potentially promoting enzymatic modification and functionalization with proteins (e.g., corona protein) present in the cytoplasm and organelles (Ma et al., 2018; Marmioli et al., 2020). ENMs may maintain crystal structure when internalized by cells or may be disassembled and converted into less complex structures (by biological modification or chelation), thus reducing toxicity, and the risk of their accumulation and translocation (Wang et al., 2022). These post-uptake structural modifications involve specific parameters such as bond distance with other atoms or nature of the ligand atoms. In consideration of this, one objective in biotransformation studies is to investigate the physico-chemical forms (e.g., nanocrystal structure) within exposed tissues and to characterize the structural differences within the new biotransformed molecules, including identification of the biomolecules interacting with the ENMs (Castillo-Michel et al., 2017; Marmioli et al., 2020). It has furthermore to consider how genetic diversity across different plant species and within the same plant species (in different cultivars) may influence the ENM uptake and translocation (Deng et al., 2020).”

New references included:

Deng C., Wang Y., Cota-Ruiz K., Reyes A., Sun Y., Peralta-Videa J.R., Hernandez-Viezcas J.A., Turley R.S., Niu G., Li C., Gardea-Torresdey J.L. Bok choy (*Brassica rapa*) grown in copper oxide nanoparticles-amended soils exhibits toxicity in a phenotype-dependent manner: Translocation, biodistribution and nutritional disturbance. *J Haz Mater*, 2020, 398, 122978.

Mitrano D.M., Motellier S., Clavaguera S., Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products, *Environment International*, 2015, 77, 132-147.

Rawat S., Pullagurala V.L.R., Adisa I.O., Wang Y., Peralta-Videa J.R., Gardea-Torresdey J.L. Factors affecting fate and transport of engineered nanomaterials in terrestrial environments, *COESH*, 2018, 6, 47-53.

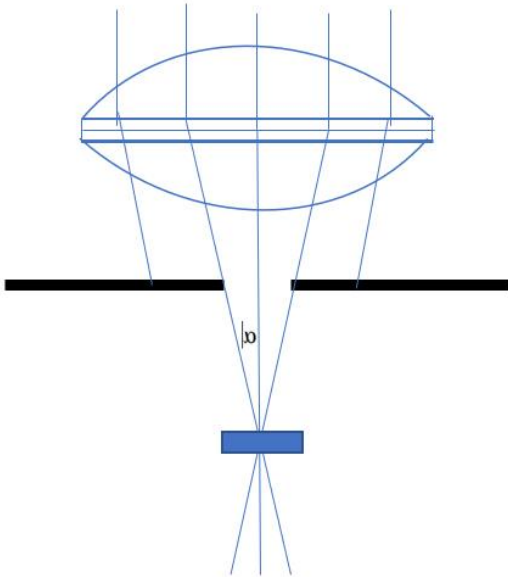
Xu T., Wang Y., Aytac Z., Zuverza-Mena N., Zhao Z., Hu X., Ng K.W., White J.C., Demokritou P. Enhancing Agrichemical Delivery and Plant Development with Biopolymer-Based Stimuli Responsive Core–Shell Nanostructures. *ACS Nano* 2022, 16, 4, 6034–6048.

*L125-147 Please add a few sentences to compare the differences between these synchrotron-based techniques and non-synchrotron-supported ones, For example, u-XRF vs. TEM-EDS. What are the advantages?*

According to the Abbe's Law the resolution power of a particle is  $d$ .

$d = \lambda / (2n \sin \alpha)$  In optical microscopy we can approximate  $d$  with  $\lambda$ .

In electron microscopy like TEM or SEM we use electrons that are accelerated passing through a potential difference they become thus equivalent to electromagnetic wave, as reported in the scheme.



Therefore, thanks to the De Broglie law we have  $\lambda(\text{nm}) = 1.22 / \sqrt{V}$ , where  $V$  is the acceleration voltage of the electrons.

In TEM  $V =$  around 30 to 100, thus  $\lambda$  varies between 0.007 nm and 0.004 nm respectively. The smallest of these resolutions are enough to see the electron lattice. With the synchrotron we use photon that do not have mass, therefore the factor 1.22 is substituted by the non-relativistic mass of the electron which is  $m = 5.485799065(16) \times 10^{-4}$  Da (according to *The NIST Reference on Constants, Units, and Uncertainty*. NIST. 20 May 2019. Retrieved 2020-06-21), this makes the resolution even smaller and increases the penetration depth into the sample.

The EDX is another thing because it depends on the acceleration voltage of the particle or of the photon. Every Element in the periodic table has its own orbitals energies, and the acceleration voltage allows to excite one or more of these, independently if it comes from a TEM or from a synchrotron. Hence in this respect the two techniques are alike. (Goldstein, Newbury et al Editors. *Scanning Electron Microscopy and X-Ray Microanalysis*, Third Edition. 2003 Springer Science+Business Media LLC, 233 Spring Street, New York, NY 10013 USA).

The text below has been added as a shortened version of these concepts.

Lines 160-165: "Synchrotron-based techniques use photons, which do not have mass; therefore the factor 1.22 is substituted by the non-relativistic mass of the electron which is  $m = 5.485 \times 10^{-4}$  Da. This makes the resolution even smaller and increases penetration depth into the sample. On the other hand, EDX depends on the acceleration voltage of the particle or of the photon. Every element has its own orbital energies, and the acceleration voltage allows excitation one or more of these, independently if it comes from a TEM or from a synchrotron (Goldstein et al., 2003)."

A new reference has been included:

Goldstein J.I., Newbury D.E., Echlin P., Joy D.C., Lyman C.E., Lifshin E., Sawyer L., Michael J.R. Editors. Scanning Electron Microscopy and X-Ray Microanalysis, Third Edition. 2003 Springer Science+Business Media LLC, 233 Spring Street, New York, NY 10013 USA.

*L195 Add nano delivery system for smart release  
[Ref]*

We thank Rev1 for the suggestion. The reference has been included and the text has been edited accordingly:

Lines 210-215: “Beyond the potential adverse effects upon bioaccumulation from soil or other exposure routes, there is an increasing interest in exploiting the potential positive effects of ENMs on plants, aiming to improve crop yields and quality. A range of mechanisms, including direct use as nanofertilizers (Verma et al., 2022), nanocarriers (Karny et al., 2018), smart delivery systems (Xu et al., 2022) or when in association to plant growth-promoting bacteria, are considered (Prado de Moraes et al., 2021).”

Xu T., Wang Y., Aytac Z., Zuverza-Mena N., Zhao Z., Hu X., Ng K.W., White J.C., Demokritou P. Enhancing Agrichemical Delivery and Plant Development with Biopolymer-Based Stimuli Responsive Core–Shell Nanostructures. ACS Nano 2022, 16, 4, 6034–6048.

*L251-267 Only one sentence was describing the synchrotron-based analysis. All the others were biomass or plant physiological responses. What does "limited stability of nLa2O3" mean? What are the results of the u-XRF analysis? Why is it unique compared to the other related analysis?*

We thank Rev1 for the comment. The sentence has been clarified.

Lines 272-275: “The limited stability of nLa<sub>2</sub>O<sub>3</sub>, as compared to nCeO<sub>2</sub>, has been confirmed by  $\mu$ -XRF analysis in *Cucumis sativus* L. through element speciation, dissolution studies in aqueous solution and *in planta*. After 14d treatment, the nCeO<sub>2</sub> structure in the roots remains mostly preserved (more than 80%) while pristine nLa<sub>2</sub>O<sub>3</sub> structure was observed at levels below 10% (Ma et al., 2015).”

Considering how Ce and La elemental properties play a fundamental role in their biotransformation, but also how their similarity in term of effects when analyzing the respective bulk forms, we believe that the direct comparison of the two nanoforms by XRF, as supported by data published in literature, could be an aspect to be described, and an interesting explanation for the physiological and molecular outcomes. Information about comparisons with other nanoforms has been reported at level of physiological and molecular analyses, both in terms of individual treatments, but also as a function of binary co-exposure (see Pagano et al., 2016, Pagano et al. 2017), which are reported in the text. This have been done with the aim to combine the potential results from physical and chemical analyses with results from physiological and molecular evidence, thereby providing a more mechanistic overview of the biotransformation processes.

*L269 Please add more recently works:  
[Ref]  
[Ref]weathering effect in soil*

The references indicated have been included and discussed.

Lines 312-318: “Interestingly, when either considering the utilization of pristine and coated nTiO<sub>2</sub> (hydrophilic or hydrophobic) in carrot (*Daucus carota* L.), responses observed depended mainly on the nTiO<sub>2</sub> surface coating, concentration and in soil weathering (Wang et al., 2021a; 2021b). Taproot and leaf fresh biomass and plant height were all increased with exposure, as well as nutrient uptake (Fe in leaves; Mg in taproots; Ca, Zn, K in roots). Conversely, sugar and starch contents were negatively affected, compromising the nutritional quality (Wang et al., 2021b).”

References included:

Wang Y., Deng C., Cota-Ruiz K., Tan W., Reyes A., Peralta-Videa J.R., Hernandez-Viezcas J.A., Li C., Gardea-Torresdey J.L. Effects of different surface-coated nTiO<sub>2</sub> on full-grown carrot plants: Impacts on root splitting, essential elements, and Ti uptake. *J Haz Mater*, 2021a, 402, 123768.

Wang Y., Deng C., Cota-Ruiz K., Peralta-Videa J.R., Hernandez-Viezcas J.A., Gardea-Torresdey J.L. Soil-aged nano titanium dioxide effects on full-grown carrot: Dose and surface-coating dependent improvements on growth and nutrient quality. *Scie Tot Environ*, 2021b, 774, 145699.

*L395-398 Please add more recent works regarding the nCuO-plant interactions. Please check:*

*[Ref]*

*[Ref](in planta biodistribution of nCuO in the particle form and growth related gene expression)*

The references indicated have been included and discussed.

Lines 452-464: “The grain of weedy and cultivated rice were differentially impacted by nCuO, bulk or ionic forms, showing also a cultivar-specific and concentration-dependent response. Cu translocation directly influenced plant yield, sugar production, starch content, protein content, and expression of auxin associated genes in grain (Deng et al., 2022b). Analyzing the effect of citric acid (CA) coated copper oxide (CA-nCuO) and its application (foliar spray or soil exposure) on the growth and physiology of soybean (*Glycine max* L.), nCuO appeared to be more accessible for plant uptake, as compared to CA-nCuO, decreasing the protein content, and inhibiting plant growth. CA reduced CuO NPs toxicity, demonstrating that surface modification may change the toxic properties of NPs (Deng et al., 2022c).

Treatment of *Lactuca sativa* L. with nCuO significantly increased biomass as compared to CuO microparticles (Wang et al., 2019). In addition, plants can benefit from nCuO treatment through enhanced defensive pathways, and through direct antimicrobial and antifungal activities (Elmer et al., 2018).”

References included:

Deng C., Wang Y., Navarro G., Sun Y., Cota-Ruiz K., Hernandez-Viezcas J.A., Niu G., Li C., White J.C., Gardea-Torresdey J. 2022b. Copper oxide (CuO) nanoparticles affect yield, nutritional quality, and auxin associated gene expression in weedy and cultivated rice (*Oryza sativa* L.) grains, *Scie Tot Environ*, 810, 152260.

Deng C., Wang Y., Cantu J.M., Valdes C., Navarro G., Cota-Ruiz K., Hernandez-Viezcas J.A., Li C., Elmer W.H., Dimkpa C.O., White J.C., Gardea-Torresdey, J.L. 2022c. Soil and foliar exposure of soybean (*Glycine max*) to Cu: Nanoparticle coating-dependent plant responses, *NanoImpact*, 26,100406.

**Reviewer #2:**

*1. Please rewrite highlights 1 and 3. The highlight should point out the most critical findings, conclusions, or perspectives in this review.*

Highlights have been modified in order to be more representative of the review text:

- Biotransformation is a fundamental phenomenon for understanding ENM-organism response mechanisms
- Synchrotron-based methodological analyses are critical for investigating ENM biotransformation
- Biotransformation of ENMs may have positive or negative effects when considering the agri-food application

*2. Half of the text in this review are talking about the synchrotron-based analyses of ENM treated plant samples. However, it was not mentioned in the title, and the abstract only mentioned it once of its "increased use".*

We thank Rev2 for the suggestions. Synchrotron-based techniques are becoming more important to comprehend the real physico-chemical forms of ENMs within plants tissues and organs and can give the missing information that we do not have from the physiological and molecular analyses, as suggested by the relevance of some of the most influential publications on this topic cited in the text (Castillo et al., 2017; Hameed et al., 2022). However, synchrotron-based techniques are not the main focus of the review. The mechanism of biotransformation behind the plant response to ENMs is the fundamental point. Synchrotron based techniques are certainly instrumental for shedding light on those mechanisms and the combination of results from physical analyses with physiological and molecular observations can give a more comprehensive picture of what happens during ENMs treatment *in planta*. We are aware of the relevance of Synchrotron based techniques and we decided to give them an appropriate space in the introduction, in the (new) paragraph 1.2.

For these reasons we believe that title represents our work in the proper manner, and we would like to maintain it in the present form, where the emphasis is more on the mechanisms, while the techniques are tools to clarify those mechanisms.

The abstract, on the other hand, has been modified on order to be more informative on the points discussed in the main text:

“Research on engineered nanomaterials (ENMs) exposure has continued to expand rapidly, with a focus on uncovering the underlying mechanisms. The EU largely limits the number and the type of organisms that can be used for experimental testing through the 3R normative. There are different routes through which ENMs can enter the soil-plant system: this includes the agricultural application of sewage sludges, and the distribution of nano-enabled agrochemicals. However, a thorough understanding of the physiological and molecular implications of ENMs dispersion and chronic low-dose exposure remains elusive, thus requiring new evidence and a more mechanistic overview of pathways and major effectors involved in plants. Plants can offer a reliable alternative to conventional model systems to elucidate the concept of ENM biotransformation within tissues and organs, as a crucial step in understanding the mechanisms of ENM-organism interaction. To facilitate the understanding of the physico-chemical forms involved in plant response, synchrotron-based techniques have added new potential perspectives in studying the interactions between ENMs and biota. These techniques are providing new insights on the interactions between ENMs and biomolecules. The present review discusses the principal outcomes for ENMs after intake by plants, including possible routes of biotransformation which make their final fate less uncertain, and therefore require further investigation.”

*3. The whole introduction (from L41-L96) is talking about the reported environmental exposure of ENM and the effects of quantum dots on plant growth. Please revise this part and be more focused on your main topic. This review aims to summarize the synchrotron-based analysis of the ENM biotransformation in plants and some molecular effects.*

We thank Rev2 for the comment. Introduction has been modified in order to be more topic oriented.

Lines 47-99: “Although global food production has generally increased over time, the distribution has been far from equitable, with more than 820 million people having insufficient food and many more consuming low-quality diets leading directly to micronutrient deficiencies (Willett et al 2019; Zhong et al., 2020). In the past 20 years, and particularly in the past decade, engineered nanomaterials (ENMs) have seen dramatically increasing use and had an equally significant impact on ecosystems services and human society. As such, studies focused on the implications associated with their use are critical. In fact, it is known that ENMs exert important, but not completely understood, effects on biota; a particular topic of concern include the effect on crops, food production, and trophic transfer (Gardea-Torresdey et al., 2014; Ma et al., 2018; White et al., 2022).

The interplay between plant growth, dissolution, evaporation, and aggregation are key aspects of the dynamic behavior of ENMs in the environment. Directional aggregation can result in the formation of larger particles with a more complex morphology. However, given the complexity of natural environments, most nanomaterials can be found in hetero-aggregated composites of different inorganic and organic materials (Judy et al., 2012; Ma et al 2018). These aggregates can be very different from original simple pristine morphologies and may even form highly branched structures similar to fractals, all of which subsequently dramatically affect their reactivity and transport (Ma et al., 2018; Huangfu et al., 2019).

Sectors with a large nanomaterial application such as medicine and food production may experience greater risks of ENMs exposure due to their uses, with thousands of tons of ENMs that are eventually discarded into the three main environmental matrices: soil, water, and air (Keller et al., 2013; Zuverza Mena et al., 2017). ENMs with the greatest historical use include nanoscale ceria (nCeO<sub>2</sub>), silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva, 2014; Mitrano et al., 2015). Considering the long-time span of use of select materials (1950 to 2050),

efforts to estimate ENMs release through commercial and associated activity into the environment have been undertaken. For example, for nSiO<sub>2</sub>, a global production between 100,000 and three million tons per year has been estimated, while for nCeO<sub>2</sub>, levels likely reach the upper limit of 10,000 tons per year, and for nAg, the literature reflects a production volume below 1,000 tons per year (Giese et al., 2018). The use of nTiO<sub>2</sub> for the inhibition of microbial proliferation in food is one of the most important ways to prolong the shelf life of packaged products (Abutalib & Rajeh., 2020). However, a panel from EFSA concluded that E171 (TiO<sub>2</sub>) can no longer be considered as safe when used as a food additive (EFSA Journal 2021).

Gottschalk et al., (2009) calculated environmental concentrations using a probabilistic LCA (life cycle analysis) of ENM containing products. The authors modelled nTiO<sub>2</sub>, nZnO, nAg, carbon nanotubes (CNT), and fullerenes for the U.S., Europe, and Switzerland. The concentrations in the environment were calculated through probabilistic density functions and compared to ecotoxicology data. In the simulations, the values ranged from 0.003 ng L<sup>-1</sup> (fullerenes) to 21 ng L<sup>-1</sup> (nTiO<sub>2</sub>) for surface waters and from 4 ng L<sup>-1</sup> for fullerenes to 4 µg L<sup>-1</sup> for nTiO<sub>2</sub> for sewage treatment effluents. In Europe and the U.S., ENMs increased annually in sludge treated soil, and ranged from 1 ng kg<sup>-1</sup> for fullerenes to 89 µg kg<sup>-1</sup> for nTiO<sub>2</sub> (Gottschalk et al. 2009; Keller et al., 2013; Keller & Lazareva, 2014; Rincon, 2019).

Importantly, quantum dots (QDs), as well as many carbon- and metal-based ENMs, have been shown to produce negative effects on animals and plants as a function of dose, including accumulation, alteration of physiological and biochemical parameters, and reduced growth or yield (Oh et al., 2016; Zuverza-Mena et al., 2017). Quantum dots have shown to enter plant roots and to damage the cell wall, dysregulating metabolism (Marmioli et al., 2020). While mercaptoacetic acid (MAA)-coated CdSe/ZnS QDs induced minimal toxicity on maize seedlings, pristine cadmium/tellurium (Cd/Te) QDs induced chromatin stress, mitochondrial damage and inhibition on green gram sprouts (*Phaseolus radiatus* L.) growth (Song et al., 2013). There are several routes by which ENM can enter the soil-plant system. These include agricultural application of sewage sludges which often contain nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, and nAg; as well as the application of nano-enabled agrochemicals, resulting in the direct entry of nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, nFeOx, nCuO, CeO<sub>2</sub> and nAg into agricultural soils (Lv et al., 2019; Verma et al., 2022).”

Additional references included:

Gardea-Torresdey J.L., Rico C.M., White J.C. Trophic transfer, transformation, and impact of engineered nanomaterials in terrestrial environments. *Environ. Sci. Technol.* 2014, 48, 2526–2540.

Keller A.A., McFerran S., Lazareva A., Suh S. 2013. Global life cycle releases of engineered nanomaterials. *J. Nanopart. Res.* 15, 1692–1709.

Keller A.A., Lazareva A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environ. Sci. Technol. Lett.* 2014, 1, 1, 65–70.

Mitrano D.M., Motellier S., Clavaguera S., Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products, *Environment International*, 2015, 77, 132-147.

Rincon A.M. 2019. Chapter 6 - Presence of nanomaterials on consumer products: food, cosmetics, and drugs. pp. 165-181. Marmioli N., White J.C., Song J., Eds: In *Micro and Nano Technologies, Exposure to Engineered Nanomaterials in the Environment*, Elsevier, Amsterdam, The Netherlands.



Song U., Jun H., Waldman B., Roh J., Kim Y., Yi J., Lee E.J. Functional analyses of nanoparticle toxicity: a comparative study of the effects of TiO<sub>2</sub> and Ag on tomatoes (*Lycopersicon esculentum*) *Ecotoxicol. Environ. Saf.*, 2013. 93, 60-67.

White J.C., Zuverza-Mena N., Elmer W.H. From nanotoxicology to nano-enabled agriculture: Following the science at the Connecticut Agricultural Experiment Station (CAES). *Plant Nano Biology*, 2022, 1, 100007. Doi: 10.1016/j.plana.2022.100007.

*4. L66 Please add more appropriate citations. Can you provide any related numbers?*

New references have been added in order to give more quantitative information:

Lines 64-70: “Sectors with a large nanomaterial application such as medicine and food production may experience greater risks of ENMs exposure due to their uses, with thousands of tons of ENMs that are eventually discarded into the three main environmental matrices: soil, water, and air (Keller et al., 2013; Zuverza Mena et al., 2017). ENMs with the greatest historical use include nanoscale ceria (nCeO<sub>2</sub>), silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva, 2014; Mitrano et al., 2015).”

New references included:

Keller A.A., McFerran S., Lazareva A., Suh S. 2013. Global life cycle releases of engineered nanomaterials. *J. Nanopart. Res.* 15, 1692–1709.

Keller A.A., Lazareva A. Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environ. Sci. Technol. Lett.* 2014, 1, 1, 65–70.

Mitrano D.M., Motellier S., Clavaguera S., Nowack B. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products, *Environment International*, 2015, 77, 132-147.

*5. L67 and L78 Please define nSiO<sub>2</sub>, nAg, nZnO, and so forth.*

We thank Rev2 for the comment. We included the definition of each ENM cited:

Lines 67-70: “ENMs with the greatest historical use include nanoscale ceria (nCeO<sub>2</sub>), silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva, 2014; Mitrano et al., 2015).”

*6. L71 Please add a citation.*

The sentence has been fixed in order to give the proper citation.

Lines 72-75: “For example, for nSiO<sub>2</sub>, a global production between 100,000 and three million tons per year has been estimated, while for nCeO<sub>2</sub>, levels likely reach the upper limit of 10,000 tons per year, and for nAg, the literature reflects a production volume below 1,000 tons per year (Giese et al., 2018).”

#### 7. L73 TiO<sub>2</sub> or nanoscale TiO<sub>2</sub>?

The typo has been fixed.

8. *You only have one subsection "1.1" in part 1. Please consider merging it into part 1. Or change it to 1.2, and put all the content before it into 1.1.*

Paragraph subdivision has been updated, including an initial subsection called “1. Engineered nanomaterial (ENM) biotransformation”. The first section is now “1.1. ENMs: from exposure to biotransformation”, while the previous section 1.1 is now shifted to 1.2.

#### 9. L101-105 Please check

[Ref]

[Ref] for factors and mechanisms of the NPs biotransformation, especially the effect of soil weathering on plant responses.

We thank Rev2 for the suggestions. The text has been updated, as reported:

Lines 102-121: “Environmental and soil physico-chemical characteristics may significantly impact on ENMs aggregation, and dissolution, which may modify ENM bioavailability, uptake, translocation, and accumulation into terrestrial plants. In fact, light and temperature may induce potential changes on the ENM structure, such as during foliar spray (Hong et al., 2021). Once within plant tissues, ENM biotransformation may alter particle stability and behaviour in terms of interactions with biomolecules, triggering differential plant defense mechanisms (Ma et al., 2018; Rawat et al., 2018). ENMs are subject to a range of processes that may lead to their partial dissolution or result in structural modifications (Milosevic et al., 2020; Marmiroli et al., 2020). A schematic representation is reported in Figure 1. Nanoparticle biotransformation is a highly complex and poorly understood series of events and has been shown to occur during weathering in the soil, trophic transfer, and translocation within plant tissues. These reactions are highly dynamic and alter the original pristine structure of the nanoparticles in a number of ways, potentially causing the release of ions, but also the consequent restructuring (or destructuring) of the nanoparticle (Servin et al., 2017a). Biotransformation of nanomaterials may rest on the interaction with biological molecules that stabilize their external reactivity, such as peptides including those involved in detoxification, (*e.g.* glutathione), fatty acids, secondary metabolites, and even components of cell membranes (Marmiroli et al., 2020). Particle properties such as size, stability, charge, and dissolution may strongly influence other biotransformation mechanisms, potentially promoting enzymatic modification and functionalization with proteins (*e.g.*, corona protein) present in the cytoplasm and organelles (Ma et al., 2018; Marmiroli et al., 2020).”

A new reference has been included, as well as an additional Figure (new Figure 1):

Hong J., Wang C., Wagner D.C., Gardea-Torresdey J.L., He F., Rico C.M. 2021. Foliar application of nanoparticles: mechanisms of absorption, transfer, and multiple impacts. *Environ. Sci.: Nano*, 8, 1196-1210.

Rawat S., Pullagurala V.L.R., Adisa I.O., Wang Y., Peralta-Videa J.R., Gardea-Torresdey J.L. Factors affecting fate and transport of engineered nanomaterials in terrestrial environments, *COESH*, 2018, 6, 47-53.

The suggested reference related to “Soil-aged nano titanium dioxide effects on full-grown carrot” has been included in the main text in the case studies reported in the paragraph 2.2 as Wang et al., 2021b.

*10. L110-113 Besides the listed possibilities, ENMs can also remain as nanoscale particles with/without the loss of surface coatings in plant tissues after being uptaken. Please check [ref]*

We thank Rev 2 for the suggestion. The reference has been cited, as reported.

Lines 121-124: “ENMs may maintain crystal structure when internalized by cells or may be disassembled and converted into less complex structures (by biological modification or chelation), thus reducing toxicity, and the risk of their accumulation and translocation (Wang et al., 2022).”

*11. L178-181 Add more latest references. Please check [ref]  
[ref]*

We thank Rev2 for the suggestions. The text has been updated, as reported:

Lines 196-199: “Studies have added significant molecular data on the effects of ENMs exposure in plants (Schwab et al., 2016; Ma et al., 2018). Different results are often observed for the same element as a function of its form or size, i.e. nanostructured, bulk, or ionic species (Pagano et al., 2016; Wang et al., 2022).”

The suggested reference related to “Copper oxide (CuO) nanoparticles affect yield, nutritional quality, and auxin associated gene expression in weedy and cultivated rice” has been included in the text in the case studies reported in the paragraph 2.6 as Deng et al., 2022b.

*12. L202 Can you briefly describe Table 1? What is Table 1 for and why are genes listed? Were they significantly affected by the ENM exposure? All the citations listed in Table 1 are related to the author themselves. Please include some works from the others.*

Genes reported in Figure 2 (former Figure 1) and Table 1 are some of those that are modulated by the different type of ENMs as reported in the cited studies. Other genes also resulted responsiveness in a ENM-specific manner during exposure and are considered for this reason as potential biomarker of exposure/effect. Moreover, these specific biomarkers are also able to testify how the modulation of the genes in the different organs is in some cases convergent between differential forms (nano, bulk, ion) of the same element. Considering that the concept of nano-specific biomarker has been introduced in some of our research groups papers, we thought to include these references in the Table

1. This was not the case of Table 2, in which papers not related to our research group on the principal evidence of the ENM biotransformation in plant observed by physiological, molecular and synchrotron-based analyses have been included.

Table 1 description has been improved as requested:

“Table 1. Genes as potential biomarkers of exposure/effect observed in roots, leaves and pollen, in different plant species (reported in Figure 2). Genes reported cannot be considered only as modulated in the different plant organs by the different type of ENMs, but also they showed a nano-specificity during the ENM response. It is also important to observe how, depending on the ENM type, biotransformation, and as indirect consequence, the transcriptomic response, can be convergent between different forms (nano, bulk, ion) of the same element (see Figure 2).”

*13. L396 According to your reference, "nanofertilizer" should be "nanofungicide or nanofertilizer" Please consider to add more recently works [ref]*

The manuscript has been modified and the citation has been included:

Lines 423-424: “Copper oxide nanomaterials (nCuO) are among the most utilized ENMs with plants, including use as a nanopesticide or nanofertilizer (Elmer et al., 2018; Lowry et al., 2019; Xu et al., 2022).”

New reference included:

Xu T., Wang Y., Aytac Z., Zuverza-Mena N., Zhao Z., Hu X., Ng K.W., White J.C., Demokritou P. Enhancing Agrichemical Delivery and Plant Development with Biopolymer-Based Stimuli Responsive Core–Shell Nanostructures. ACS Nano 2022, 16, 4, 6034–6048.

*14. L499-500 What kinds of "physical evidence" were obtained through synchrotron-based techniques? What is missing in the current studies?*

The sentence has been improved in order to clarify those physical evidence that can be retrieved through synchrotron-based techniques:

Lines 536-541: “Integration of the information from physiological and molecular analyses with physical evidence (e.g., types and number of atoms surrounding the ENM, radial distance between atoms of the interactors and atoms constituting the ENM; ENM crystal structure) obtained through high energy X-ray spectroscopy platforms such as synchrotron-based techniques will enable a more realistic, mechanistic, and systems-level picture of plant response to ENM exposure.”

*15. Figure: Only one figure was provided in the main text regarding the list of genes being affected by the ENMs. But the main focus of this review is not ENMs affected gene expressions. What happens with ENMs biotransformation and synchrotron analysis?*

We thank Rev2 for the comment. We decided to include a new Figure 1, described in the new paragraph 1.2, which schematized the major modifications that may occur during ENMs-soil-plant

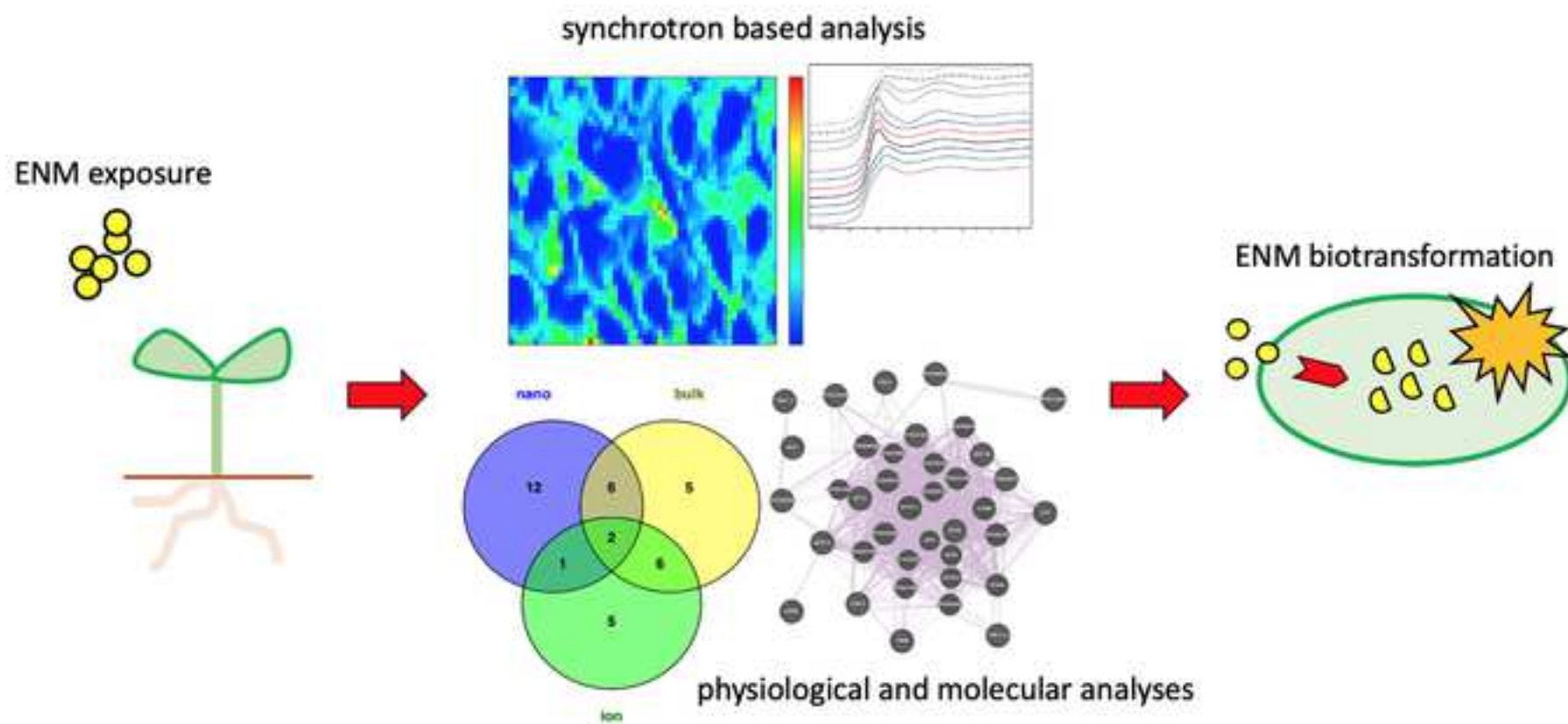
interaction. In order to maximize the comprehension of the new Figure 1, a new caption was constructed to explain in details of these modifications and their practical effects during each phase (soil, foliar surface, plant).

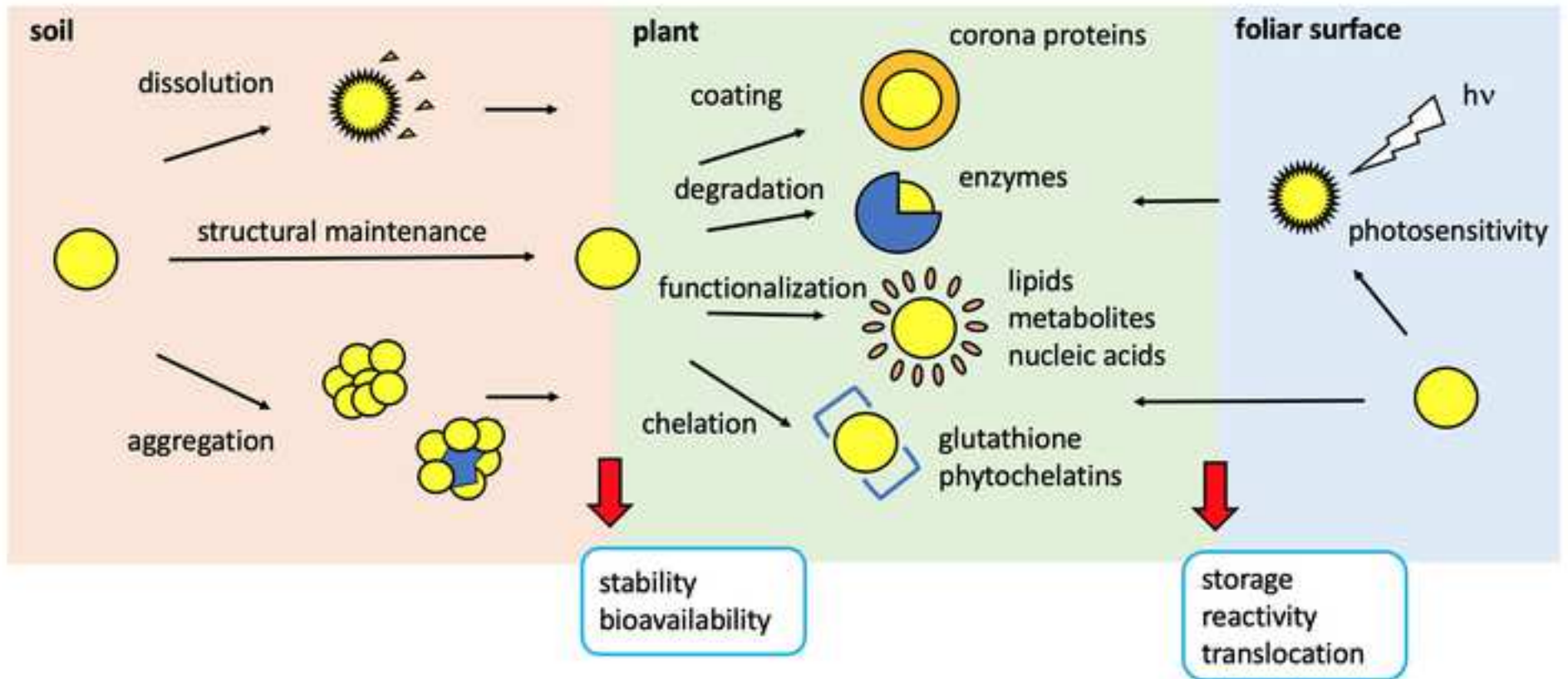
New Figure 1 caption:

“Figure 1. Schematic representation of principal effects emerging after biotransformation of ENMs in soil, on foliar surface and within the plant tissues. ENMs in soil, due to particle and soil physico-chemical interactions, may remain unmodified, or may undergo to potential dissolution (with ions release), or undergo homo-/hetero-aggregation, which may highly influence particle stability and potential bioavailability (and consequently their uptake). On the foliar surface, temperature and light may also affect particle stability before uptake into the leaf tissues. Once within the plant, ENMs pristine, or modified, can interact with several biomolecule types (peptides, sugars, lipids, nucleic acids, secondary metabolites) leading to phenomena such as coating, enzymatic degradation, chelation or functionalization, which may influence the biotransformed particle at level of translocation, storage or reactivity. These parameters may also influence the interaction within the plant cell, triggering differential responses (*e.g.*, toxicity, oxidative stress, ROS production), which may be indirectly measured by physiological and molecular assays, but directly observed through physical strategies, including synchrotron-based methods.”

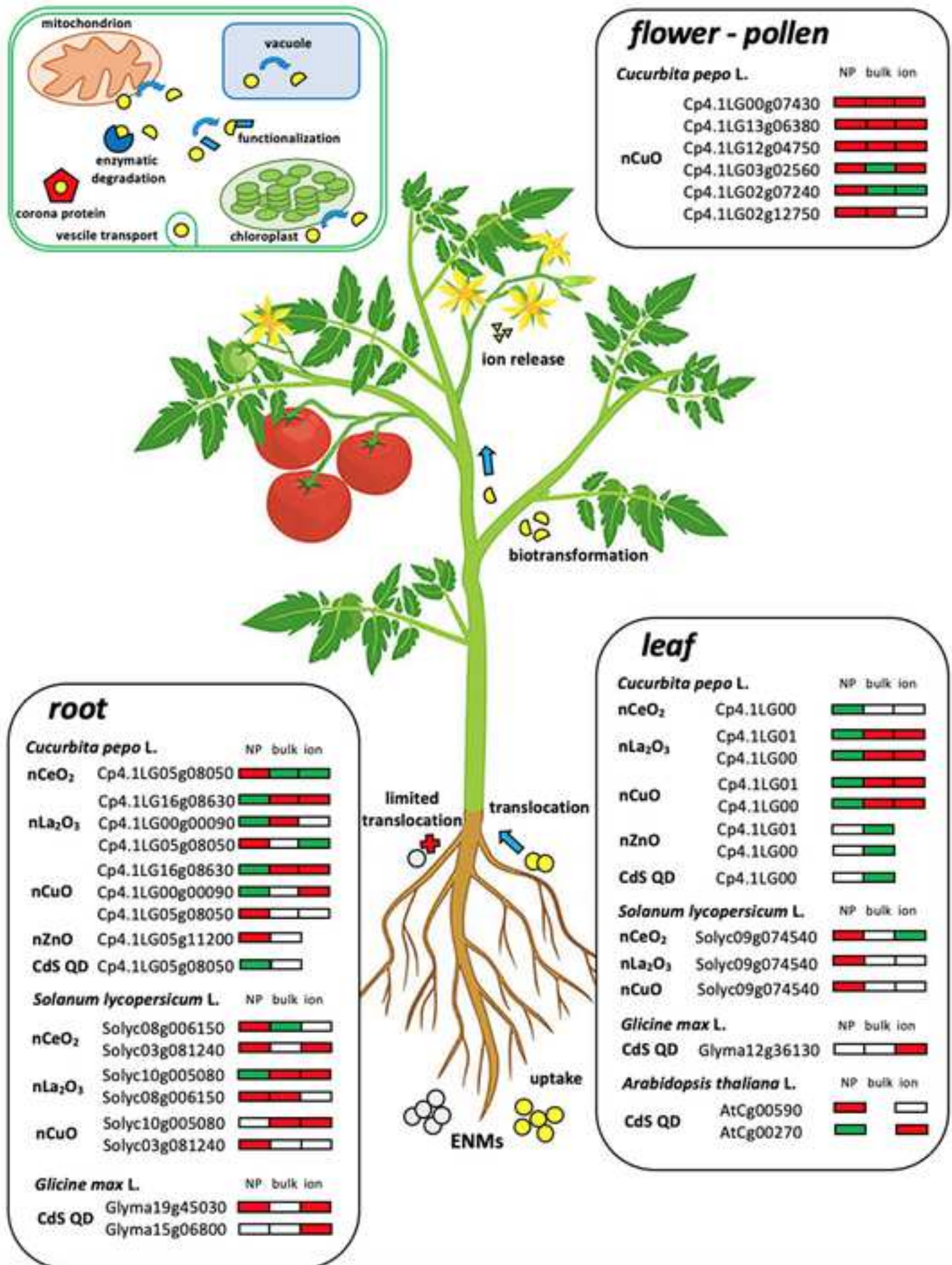
## **Highlights**

- Biotransformation is a fundamental phenomenon for understanding ENM-organism response mechanisms
- Synchrotron-based methodological analyses are critical for investigating ENM biotransformation
- Biotransformation of ENMs may have positive or negative effects when considering the agri-food application









**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Nelson Marmiroli reports financial support was provided by European Union.

Conceptualization: LP, MM; original draft preparation: LP, MM, RR; review and editing: JCW, NM. All authors revised and agreed on the final version of the manuscript.

## 1 **Nanomaterials biotransformation: In planta mechanisms of action**

2

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4

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15

### 16 **Abstract**

17 Research on engineered nanomaterials (ENMs) exposure has continued to expand rapidly, with a  
18 focus on uncovering the underlying mechanisms. The EU largely limits the number and the type of  
19 organisms that can be used for experimental testing through the 3R normative. There are different  
20 routes through which ENMs can enter the soil-plant system: this includes the agricultural application  
21 of sewage sludges, and the distribution of nano-enabled agrochemicals. However, a thorough  
22 understanding of the physiological and molecular implications of ENMs dispersion and chronic low-  
23 dose exposure remains elusive, thus requiring new evidence and a more mechanistic overview of  
24 pathways and major effectors involved in plants. Plants can offer a reliable alternative to conventional  
25 model systems to elucidate the concept of ENM biotransformation within tissues and organs, as a  
26 crucial step in understanding the mechanisms of ENM-organism interaction. To facilitate the

27 understanding of the physico-chemical forms involved in plant response, synchrotron-based  
28 techniques have added new potential perspectives in studying the interactions between ENMs and  
29 biota. These techniques are providing new insights on the interactions between ENMs and  
30 biomolecules. The present review discusses the principal outcomes for ENMs after intake by plants,  
31 including possible routes of biotransformation which make their final fate less uncertain, and  
32 therefore require further investigation.Research on engineered nanomaterials (ENMs) exposure has  
33 continued to expand rapidly, and with studies focused on uncovering underlying mechanisms are  
34 concerned, EU largely limits the number and the type of organisms that can be used so as to support  
35 the 3R normative. There are different routes by which ENMs can enter the soil-plant system: this  
36 includes the agricultural application of sewage sludges, and the application of nano enabled  
37 agrochemicals, resulting in the direct entry of ENMs into agricultural soil. However, a thorough  
38 understanding of the physiological and molecular implications of chronic low dose exposure to  
39 engineered nanomaterials remains elusive. Plants can offer a reliable alternative and, in this context,  
40 the concept of nanomaterial biotransformation within plant tissues and organs is one of increasing  
41 interest. To facilitate understanding of the physico-chemical forms involved in plant response,  
42 synchrotron based techniques have seen increasing use and have added new perspectives on the  
43 interactions between ENMs and biota. The review will discuss the principal outcomes related to the  
44 ENMs biotransformation in plants and the practical relevance of those findings, as well as the  
45 potential applicability of those findings to other biotic species.

46  
47 **Keywords:** biotransformation, plant, nanomaterials, synchrotron-based analyses, molecular response

48  
49 **Highlights**

- 50 • Biotransformation is a fundamental phenomenon for understanding ENM-organism response  
51 mechanisms

52 • Synchrotron-based methodological analyses are critical for investigating ENM  
53 biotransformation

54 • Biotransformation of ENMs may have positive or negative effects when considering the agri-  
55 food application

56 • Biotransformation is fundamental to understanding ENM-organism response

57 • Synchrotron-based analyses are critical to investigate ENM biotransformation

58 • Understanding ENM biotransformation is critical to safer by design applications

59

60

61 **1. Engineered nanomaterial (ENM) biotransformation**

62 ~~From ENM exposure to biotransformation~~ ENMs: from *From ENM exposure to*  
63 *biotransformation*

64 **1.1.1.**

65 ~~More than three billion metric tons of crops are produced globally each year in the world, requiring~~  
66 ~~187 million metric tons of fertilizer, nearly 4 million tons of pesticides, 2.7 trillion cubic meters of~~  
67 ~~water (about 70% of all freshwater consumptive use globally), and over two quadrillion British~~  
68 ~~thermal units (BTU) of embodied energy (Lowry et al., 2019; Zhong et al., 2020). Although the~~  
69 ~~benefits of the Green Revolution have enabled consumption of an average 2,884 kcal per capita per~~  
70 ~~day in the WHO Countries, conventional agricultural practices are unsustainable, and have directly~~  
71 ~~led to significant environmental damage (Willett et al., 2019). Although global food production of~~  
72 ~~calories has generally kept pace with population growth, the distribution has been far from equitable,~~  
73 ~~with more than 820 million people having insufficient food and many more consuming low quality~~  
74 ~~diets leading directly micronutrient deficiencies (Willett et al 2019). In the past 20 years, and~~  
75 ~~particularly in the past decade, engineered nanomaterials (ENMs) have seen dramatically increasing~~  
76 ~~use and have had equally significant impact on ecosystems and human society; as such, studies~~  
77 ~~focused on the implications associated with that use are critical. In fact, it is known that nanomaterials~~

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78 exert important, but often poorly understood, impacts on biota; particular topics of concern include  
79 human health, agriculture and food production (Ma et al., 2018).

80 The interplay between growth, dissolution, evaporation, and aggregation are key aspects of the  
81 dynamic behavior of nanomaterials in the environment. Directional aggregation can result in the  
82 formation of larger particles with complex morphologies. However, given the complexity of natural  
83 environments, most nanomaterials are found in heteroaggregated composites of different inorganic  
84 and organic materials (Judy et al., 2012; Ma et al 2018). These aggregates can be very different from  
85 original simple pristine morphologies and may even form highly branched structures similar to  
86 fractals, all of which subsequently dramatically affects reactivity and transport properties (Ma et al.,  
87 2018; Huangfu et al., 2019).

88 Sectors with widespread nanomaterial application such as medicine and food production carry a  
89 greater risk of ENMs exposure, and associated with these uses is the thousands of tons of ENMs that  
90 are discarded into the three main environmental matrices of soil, water, and air (Zuverza Mena et al.,  
91 2017). The ENMs with the greatest historical use include nanoscale  $\text{CeO}_2$  (nCeO<sub>2</sub>), nSiO<sub>2</sub>, and nAg,  
92 and as such, release in the environment has been investigated. Given the long time span of use of  
93 select materials (1950 to 2050), efforts to estimate ENMs release through commercial and associated  
94 activity into the environment have been undertaken. For example, for nSiO<sub>2</sub>, global production  
95 between 100,000 and about three million tons per year has been estimated. For nCeO<sub>2</sub>, levels likely  
96 reach the upper limit of 10,000 tons per year, and for nAg, the literature reflects a production volume  
97 below 1,000 tons per year (Giese et al., 2018). Use of TiO<sub>2</sub> for inhibition of microbial proliferation  
98 in food is one of the most important ways to prolong the shelf life of packaged products (Abutalib &  
99 Rajeh., 2020). In addition, a panel from EFSA concluded that E171 can no longer be considered as  
100 safe when used as a food additive (EFSA Journal 2021).

101 Gottschalk et al., (2009) calculated environmental concentrations using a probabilistic life-cycle  
102 analysis of ENM containing products. The authors modelled nTiO<sub>2</sub>, nZnO, nAg, carbon nanotubes  
103 (CNT), and fullerenes for the U.S., Europe, and Switzerland. The concentrations in the environment

104 were calculated through probabilistic density functions and compared to ecotoxicology data. In the  
105 simulations, the values ranged from 0.003 ng L<sup>-1</sup> (fullerenes) to 21 ng L<sup>-1</sup> (nTiO<sub>2</sub>) for surface waters  
106 and from 4 ng L<sup>-1</sup> for fullerenes, to 4 µg L<sup>-1</sup> for nTiO<sub>2</sub> for sewage treatment effluents. In Europe and  
107 the U.S., ENMs increased annually in sludge-treated soil, and ranged from 1 ng kg<sup>-1</sup> for fullerenes to  
108 89 µg kg<sup>-1</sup> for nTiO<sub>2</sub> (Gottschalk et al. 2009).

109 Importantly, quantum dots (QDs), as well as many carbon- and metal-based ENMs have been shown  
110 to produce negative effects on animals and plants as a function of dose, including accumulation,  
111 alteration of physiological and biochemical traits, and reduced growth or yield (Oh et al., 2016;  
112 Zuverza-Mena et al., 2017). Quantum dots have been shown to enter plant roots and to damage the  
113 cell wall and dysregulate metabolism (Marmioli et al. 2020). While mercaptoacetic acid (MAA)-  
114 coated CdSe/ZnS QDs induced minimal toxicity on maize seedlings, pristine cadmium/tellurium  
115 (Cd/Te) QDs induced chromatin stress, mitochondrial damage and inhibition in green gram sprouts  
116 (*Phaseolus radiatus* L.) growth (Zuverza-Mena et al., 2017). There are several routes by which ENM

117 can enter the soil-plant system. This includes the agricultural application of sewage sludges which  
118 often contain nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, and nAg; as well as the application of nano-enabled  
119 agrochemicals. Although global food production has generally increased over time, the distribution  
120 has been far from equitable, with more than 820 million people having insufficient food and many  
121 more consuming low-quality diets leading directly to micronutrient deficiencies (Willett et al. 2019;  
122 Zhong et al., 2020). In the past 20 years, and particularly in the past decade, engineered nanomaterials  
123 (ENMs) have seen dramatically increasing use and had an equally significant impact on ecosystems  
124 services and human society. As such, studies focused on the implications associated with their use  
125 are critical. In fact, it is known that ENMs exert important, but not completely understood, effects on  
126 biota; a particular topic of concern include the effect on crops, food production, and trophic transfer  
127 (Gardea-Torresdey et al., 2014; Ma et al., 2018; White et al., 2022).

128 The interplay between plant growth, dissolution, evaporation, and aggregation are key aspects of the  
129 dynamic behavior of ENMs in the environment. Directional aggregation can result in the formation

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130 of larger particles with a more complex morphology. However, given the complexity of natural  
131 environments, most nanomaterials can be found in hetero-aggregated composites of different  
132 inorganic and organic materials (Judy et al., 2012; Ma et al 2018). These aggregates can be very  
133 different from original simple pristine morphologies and may even form highly branched structures  
134 similar to fractals, all of which subsequently dramatically affect their reactivity and transport (Ma et  
135 al., 2018; Huangfu et al., 2019).

136 Sectors with a large nanomaterial application such as medicine and food production may experience  
137 greater risks of ENMs exposure due to their uses, with thousands of tons of ENMs that are eventually  
138 discarded into the three main environmental matrices: soil, water, and air (Keller et al., 2013;  
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140 silica (nSiO<sub>2</sub>), titania (nTiO<sub>2</sub>), as well as nanoscale copper oxide (nCuO), zinc oxide (nZnO) and  
141 nanosilver (nAg), and as such, release in the environment has been investigated (Keller & Lazareva,  
142 2014; Mitrano et al., 2015). Considering the long-time span of use of select materials (1950 to 2050),  
143 efforts to estimate ENMs release through commercial and associated activity into the environment  
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145 tons per year has been estimated, while for nCeO<sub>2</sub>, levels likely reach the upper limit of 10,000 tons  
146 per year, and for nAg, the literature reflects a production volume below 1,000 tons per year (Giese et  
147 al., 2018). The use of nTiO<sub>2</sub> for the inhibition of microbial proliferation in food is one of the most  
148 important ways to prolong the shelf life of packaged products (Abutalib & Rajeh., 2020). However,  
149 a panel from EFSA concluded that E171 (TiO<sub>2</sub>) can no longer be considered as safe when used as a  
150 food additive (EFSA Journal 2021).

151 Gottschalk et al., (2009) calculated environmental concentrations using a probabilistic LCA (life  
152 cycle analysis) of ENM containing products. The authors modelled nTiO<sub>2</sub>, nZnO, nAg, carbon  
153 nanotubes (CNT), and fullerenes for the U.S., Europe, and Switzerland. The concentrations in the  
154 environment were calculated through probabilistic density functions and compared to ecotoxicology  
155 data. In the simulations, the values ranged from 0.003 ng L<sup>-1</sup> (fullerenes) to 21 ng L<sup>-1</sup> (nTiO<sub>2</sub>) for

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156 surface waters and from 4 ng L<sup>-1</sup> for fullerenes to 4 µg L<sup>-1</sup> for nTiO<sub>2</sub> for sewage treatment effluents.  
157 In Europe and the U.S., ENMs increased annually in sludge treated soil, and ranged from 1 ng kg<sup>-1</sup>  
158 for fullerenes to 89 µg kg<sup>-1</sup> for nTiO<sub>2</sub> (Gottschalk et al. 2009; Keller et al., 2013; Keller & Lazareva,  
159 2014; Rincon, 2019).  
160 Importantly, quantum dots (QDs), as well as many carbon- and metal-based ENMs, have been shown  
161 to produce negative effects on animals and plants as a function of dose, including accumulation,  
162 alteration of physiological and biochemical parameters, and reduced growth or yield (Oh et al., 2016;  
163 Zuverza-Mena et al., 2017). Quantum dots have shown to enter plant roots and to damage the cell  
164 wall, dysregulating metabolism (Marmioli et al., 2020). While mercaptoacetic acid (MAA)-coated  
165 CdSe/ZnS QDs induced minimal toxicity on maize seedlings, pristine cadmium/tellurium (Cd/Te)  
166 QDs induced chromatin stress, mitochondrial damage and inhibition on green gram sprouts  
167 (*Phaseolus radiatus* L.) growth (Song et al., 2013). There are several routes by which ENM can enter  
168 the soil-plant system. These include agricultural application of sewage sludges which often contain  
169 nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, and nAg; as well as the application of nano-enabled agrochemicals, resulting in  
170 the direct entry of nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, nFeOx, nCuO, CeO<sub>2</sub> and nAg into agricultural soils (Lv et  
171 al., 2019; Verma et al., 2022).

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### 173 4.1.2. *Conceiving and studying the ENM biotransformation*

174 Environmental and soil physico-chemical characteristics may significantly impact on ENMs  
175 aggregation, and dissolution, which may modify ENM bioavailability, uptake, translocation, and  
176 accumulation into terrestrial plants. In fact, light and temperature may induce potential changes on  
177 the ENM structure, such as during foliar spray (Hong et al., 2021). Once within plant tissues, ENM  
178 biotransformation may alter particle stability and behaviour in terms of interactions with  
179 biomolecules, triggering differential plant defense mechanisms (Ma et al., 2018; Rawat et al., 2018).  
180 ENMs are subject to a range of processes that may lead to their partial dissolution or result in  
181 structural modifications (Milosevic et al., 2020; Marmioli et al., 2020). A schematic representation

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182 ~~is reported in Figure 1. Once accumulated by plants, ENMs are subject to a range of processes that~~  
183 ~~may induce partial dissolution or result in structural modifications (Milosevic et al., 2020; Marmioli~~  
184 ~~et al., 2020). Nanoparticle biotransformation is a highly complex and poorly understood series of~~  
185 ~~events and has been shown to occur during weathering in the soil, trophic transfer, and translocation~~  
186 ~~within plant tissues. These reactions are highly dynamic and alter the original pristine structure of the~~  
187 ~~nanoparticles in a number of ways, potentially causing the release of ions, but also the consequent~~  
188 ~~restructuring (or destructuring) of the nanoparticle (Servin et al., 2017a). Biotransformation of~~  
189 ~~nanomaterials may rest on the interaction with biological molecules that stabilize their external~~  
190 ~~reactivity, such as peptides including those involved in detoxification, (e.g., glutathione), fatty acids,~~  
191 ~~secondary metabolites, and even components of cell membranes (Marmioli et al., 2020). Particle~~  
192 ~~properties such as size, stability, charge, and dissolution may strongly influence other~~  
193 ~~biotransformation mechanisms, potentially promoting enzymatic modification and functionalization~~  
194 ~~with proteins (e.g., corona protein) present in the cytoplasm and organelles (Ma et al., 2018;~~  
195 ~~Marmioli et al., 2020). ENMs may maintain crystal structure when internalized by cells or may be~~  
196 ~~disassembled and converted into less complex structures (by biological modification or chelation),~~  
197 ~~thus reducing toxicity, and the risk of their accumulation and translocation (Wang et al., 2022). These~~  
198 ~~post-uptake structural modifications involve specific parameters such as bond distance with other~~  
199 ~~atoms or nature of the ligand atoms. In consideration of this, one objective in biotransformation~~  
200 ~~studies is to investigate the physico-chemical forms (e.g., nanocrystal structure) within exposed~~  
201 ~~tissues and to characterize the structural differences within the new biotransformed molecules,~~  
202 ~~including identification of the biomolecules interacting with the ENMs (Castillo-Michel et al., 2017;~~  
203 ~~Marmioli et al., 2020). Nanoparticle biotransformation is highly complex and poorly understood~~  
204 ~~series of reactions and has been shown to occur during soil weathering, trophic transfer, translocation~~  
205 ~~within plant tissues; these reactions are highly dynamic and alter the original pristine structure of the~~  
206 ~~nanoparticles in a myriad of ways, potentially causing the release of ions but also the consequent~~  
207 ~~restructuring (or destructuring) of the nanoparticle physico-chemical form (Servin et al., 2017a).~~

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208 ~~Biotransformation of nanomaterials may lead to the attachment of biological molecules that stabilize~~  
209 ~~their external reactivity, such as proteins, fatty acids, secondary metabolites, and even cell membranes~~  
210 ~~(Marmiroli et al., 2020). Particle properties such as size, stability, charge, and dissolution influence~~  
211 ~~the biotransformation mechanisms, potentially promoting enzymatic modification and~~  
212 ~~functionalization with proteins (e.g., corona protein) present in the cytoplasm and organelles~~  
213 ~~(Marmiroli et al., 2020). Importantly, ENMs may maintain crystal structure when internalized by~~  
214 ~~cells or may be disassembled and transformed into less complex structures (by biological~~  
215 ~~modification or chelation), thus minimizing toxicity and influencing the risk of their accumulation~~  
216 ~~and translocation. These post uptake structural modifications involve specific parameters such as~~  
217 ~~bond distance with other atoms or nature of the ligand atoms. In consideration of this, one objective~~  
218 ~~in biotransformation studies is to investigate the physico-chemical forms (e.g., nanocrystal structure)~~  
219 ~~within exposed tissues and to characterize the structural differences evident in the new~~  
220 ~~biotransformed molecules, including identification of the key biomolecules interacting with the ENM~~  
221 ~~(Castillo-Michel et al., 2017; Marmiroli et al., 2020). It has furthermore to consider how genetic~~  
222 ~~diversity across different plant species and within the same plant species (in different cultivars) may~~  
223 ~~influence the ENM uptake and translocation (Deng et al., 2020).~~

224 Some of the more interesting discoveries on the biotransformation and localization of metal based-  
225 ENMs into plants have been achieved with to the synchrotron-based techniques of imaging, elemental  
226 speciation, and atomic neighbors' identification. This transformation will be also influenced by the  
227 environment, these reactions of the same particle will be different on the leaf surface, in the  
228 mesophyll, in the vascular tissue, in different organelles, in the roots and in the rhizosphere (Sarret et  
229 al. 2013; Castillo-Michel et al., 2017).

230 Micro-X-Ray Fluorescence ( $\mu$ -XRF) and micro-X-Ray Adsorption Spectroscopy ( $\mu$ -XAS) K-, L- or  
231  $L_{III}$ -edge EXAFS and XANES spectra have been used to study the biotransformation of coated  
232 nanomaterials present in plants and soil (Lopez-Moreno et al., 2010; Judy et al., 2012).  $\mu$ -XRF is  
233 used for qualitative elemental analysis of heterogeneous biological samples. The interaction of the

234 sample with high-energy X-ray radiation, which leads to X-ray absorption and emission of the  
235 fingerprint X-ray spectra for each element, is the key feature of this powerful analytical method. The  
236 absorption/excitation effect and relaxation process lead to atoms emitting fluorescence photons  
237 characterized by elemental specific energy (Chebakova et al., 2021). Extended X-ray absorption fine  
238 structure (EXAFS) is a technique that utilizes oscillations of the X-ray absorption coefficient on the  
239 high-energy side of an absorption edge. Such oscillations can reach up to 1000 eV above the edge  
240 and may have a magnitude of 10% or more. In addition, it is necessary to consider that atoms are not  
241 stationary. Thermal vibrations will obscure the EXAFS oscillations, and in the harmonic  
242 approximation, this can be accounted for by considering a Debye-Waller-type term. This led to a  
243 great improvement in the theoretical understanding of EXAFS and it is now established that a single  
244 scattering short-range order theory is adequate under most circumstances (Gurman, 1995). In  
245 addition, electrons that have undergone inelastic losses will not interference in the process. This is  
246 considered by an exponential damping term. It is the limited range of the photoelectrons in the energy  
247 region of interest 50-1000 eV that allows for a short-range order description of EXAFS also in  
248 crystalline materials (Gurman, 1995). The region which includes the pre-edge, edge-jump and post-  
249 edge to approximately 30-50 eV is referred to as the X-ray Absorption Near Edge Structure  
250 (XANES), which describes the structural component of the X-ray absorption near-edge as an  
251 extension of the EXAFS, largely due to the long mean free path of the photoelectron and the  
252 dominance of high order multiple scattering contributions (Gräfe et al., 2014).

253 Synchrotron-based techniques use photons, which do not have mass; therefore the factor 1.22 is  
254 substituted by the non-relativistic mass of the electron which is  $m=5.485 \cdot 10^{-4}$  Da. This makes the  
255 resolution even smaller and increases penetration depth into the sample. On the other hand, EDX  
256 depends on the acceleration voltage of the particle or of the photon. Every element has its own orbital  
257 energies, and the acceleration voltage allows excitation one or more of these, independently if it  
258 comes from a TEM or from a synchrotron (Goldstein et al., 2003).

259 From the perspective of application,  $\mu$ -XRF can provide information on the presence and localization  
260 of specific elements within tissues, while XANES and EXAFS spectroscopy can provide information  
261 related to the valence state and coordination environment of the element of interest, as well as the  
262 molecular species present in the sample. The use of  $\mu$ -XRF and  $\mu$ -XANES for the analysis of  
263 nanoparticles in plants have been thoroughly reviewed by Castillo-Michel et al. (2017).  
264 Importantly, these powerful methodologies open the possibility to mechanistically address many  
265 important environmental issues, such as the chemical activities of environmental pollutants, to trace  
266 environmental elemental cycles, element speciation in complex matrices, and to characterize the  
267 natural/anthropogenic complex matrixes that are not amendable for standard analytical and structural  
268 analyses (Puri et al., 2019). In many synchrotrons around the world, there is increasing use of  
269 dedicated beamlines to study environmental and plant tissues exposed to contaminants such as ENMs  
270 (Proux et al., 2017; d'Acapito et al., 2019).

271

272 ~~This~~ present review aims to describe the current understanding of metal based-ENMs  
273 biotransformation mechanisms in ~~plants, and plants and~~ focuses on correlating available physiological  
274 and molecular data with the information obtained by synchrotron-based techniques. This evaluation  
275 not only highlights biotransformation as one of the major driving forces mediating the biological  
276 effects of ENMs on plants, but also offers some perspective on intentional and safer-by-design  
277 strategies that can ensure more sustainable application of these materials. Moreover, the study on  
278 plants is instrumental to the application of the REACH normative within European Union for  
279 toxicological and ecotoxicological studies (Replacement, Reduction and Refinement). Plants are  
280 higher eukaryotes, characterized by large nuclear genomes and organellar genomic information  
281 (within chloroplasts and mitochondria) that provide an effective model for many complex species  
282 (Chang et al., 2016).

283

284

## 285 *2. Physiological and molecular effects as indirect evidence of ENM biotransformation in plants*

286 The physiological behaviour and related molecular pathways of response are important to  
287 characterizing and understanding ENM biotransformation. An important part of this involves  
288 comparing the effects of a nanomaterial with that of the bulk and ionic counterparts, as well as by  
289 investigating different exposure times and by exposing different plant organs and tissues (Schwab et  
290 al., 2016; Marmiroli et al., 2021). Studies have added significant molecular data on the effects of  
291 ENMs exposure in plants (Schwab et al., 2016; Ma et al., 2018). Different results are often observed  
292 for the same element as a function of its form or size, i.e. nanostructured, bulk, or ionic species  
293 (Pagano et al., 2016; Wang et al., 2022).~~A number of important studies have added significant~~  
294 ~~molecular understanding to the effects of ENMs exposure in plants (Schwab et al., 2016; Ma et al.,~~  
295 ~~2018). Different results are often observed for the same element as a function of form or size; i.e.~~  
296 ~~nanostructured, bulk, or ionic species (Pagano et al., 2016).~~ Detailed study of differential  
297 transcriptional regulation, protein abundance or metabolomic profiling (Huang et al., 2018;  
298 Majumdar et al., 2019; Gallo et al., 2021) are critical to demonstrating the nano-specificity of plant  
299 response. The study of organellar genome stability and the related stoichiometric variations during  
300 ENM treatment has also provided important mechanistic insight into plant to ENM exposure (Pagano  
301 et al., 2022). Advanced synchrotron-based techniques may either help to systematically understand  
302 the nano-bio interactions, with regard to physical and chemical reactions at the biomolecular surface:  
303 biomolecules may interact with ENMs, generating biomolecular corona, which change the ENM  
304 surface properties, and interfere with its functionality/reactivity (Hameed et al., 2022).  
305 Regarding the physiological effects of ENMs on plants of agronomic interest, these studies have  
306 provided a better understanding of the specific properties of the ENMs that may enable sustainable  
307 use in the agrifood sector. Beyond the potential adverse effects upon bioaccumulation from soil or  
308 other exposure routes, there is an increasing interest in exploiting the potential positive effects of  
309 ENMs on plants, aiming to improve crop yields and quality. A range of mechanisms, including direct  
310 use as nanofertilizers (Verma et al., 2022), nanocarriers (Karny et al., 2018), smart delivery systems

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§11 ~~(Xu et al., 2022) or when in association to plant growth-promoting bacteria, are considered (Prado de~~  
§12 ~~Moraes et al., 2021). Beyond the potential adverse effects upon accumulation from the soil or other~~  
§13 ~~exposure routes, there is increasing interest in exploring the potential positive effects of nanomaterials~~  
§14 ~~on plants, aiming to improve crop yields and productivity by a range of mechanisms, including direct~~  
§15 ~~use as nanofertilizers (Verma et al., 2022), nanocarriers (Karny et al., 2018), or when in association~~  
§16 ~~to plant growth promoting bacteria (Prado de Moraes et al., 2021).~~ In addition, ENMs may act  
§17 indirectly by protecting plants from biotic (*e.g.*, nanopesticides) or abiotic stressors (*e.g.*, wastewater  
§18 and soil treatment) (Liu et al., 2015; Kah et al., 2018; Kumari et al., 2019). Due to the many variables  
§19 involved, it is essential to obtain robust safety data regardless of the end use: ENM type, the modes  
§20 and time of exposure, concentrations tested, and the plants used are all important considerations. Any  
§21 recurring effects that occur under these different conditions are of particular interest and are explored  
§22 below (see Figure 24 and Tables 1). Table 2 summarizes the major outcomes related to metal based-  
§23 ENM biotransformation in plants, including the principal mechanisms involved, and the major  
§24 physiological and molecular insights observed from exposure.

### §26 2.1. *Lanthanides based ENMs*

§27 Cerium Oxide (nCeO<sub>2</sub>) has shown significant potential for agricultural applications, largely due to its  
§28 properties as an ROS scavenger (Ma et al., 2015; Servin et al., 2017b). While CeO<sub>2</sub> as a bulk crystal  
§29 mainly consists of Ce(IV), the reduction to nCeO<sub>2</sub> significantly enhances the relative amount of  
§30 Ce(III), resulting in a higher catalytic effects comparable to the capabilities of a biological antioxidant  
§31 (Eriksson et al., 2018). Servin et al. (2017b) used  $\mu$ -XRF and  $\mu$ -XANES to analyze the interactions  
§32 between nCeO<sub>2</sub> and different biochars in soil, observing that much of the Ce remained in nCeO<sub>2</sub> form  
§33 within the plant tissues. The dissolution rate of the nanoform can increase in acidic environments to  
§34 generate Ce(III), as reported by Hernandez-Viezcas et al. (2013) who analyzed in *Glycine max* L. the  
§35 effects of nCeO<sub>2</sub> (1000 mg L<sup>-1</sup>, 48d exposure). Results have been confirmed by Rui et al. (2015), who  
§36 used XANES on exposed cucumber (*Cucumis sativus* L.) tissues (2000 mg L<sup>-1</sup>, 21d exposure) to

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337 observe nCeO<sub>2</sub> association with phosphate. These properties highly impact not only reactivity but  
338 also nCeO<sub>2</sub> translocation. In zucchini (*Cucurbita pepo* L.), treated with 500 mg L<sup>-1</sup> of nCeO<sub>2</sub>, the  
339 nanoform is mainly present in the roots and stems, with limited translocation to the leaves (Pagano et  
340 al., 2016). However, co-contamination with other ENMs (*e.g.*, CdS QDs) under same experimental  
341 conditions resulted in increased translocation to the shoots from 1000 to 3000 mg kg<sup>-1</sup> (Pagano et al.,  
342 2017). Similar results have been reported in several plant species: for example, Rossi et al (2017)  
343 nCeO<sub>2</sub> under co-exposure with ionic Cd in soybean (*Glycine max* L.) showed an altered (1-2 fold  
344 increased) translocation to the shoots. Interestingly, bulk CeO<sub>2</sub> translocation resulted similar to the  
345 nano-form, whereas ionic Ce was translocated in greater amounts to the shoots (Pagano et al., 2016).  
346 This analysis was supplemented with molecular data; the transcriptional profiles were evaluated in  
347 *C. pepo* and *S. lycopersicum* as a function of nCeO<sub>2</sub>, nLa<sub>2</sub>O<sub>3</sub> and nCuO exposure and were compared  
348 with bulk and ionic forms using a set of 38 genes based on the *A. thaliana* orthologs as potential  
349 biomarkers of exposure/effects (Marmioli et al., 2014). The responses observed were generally  
350 different in term of up- or down-regulation as a function of Ce form (Pagano et al., 2016). Of  
351 particular interest are impacts on the chloroplast are the *PetL* and *PSBN* genes, which encode for a  
352 structural component of the cytochrome b<sub>6</sub>f complex and low molecular weight protein located on  
353 thylakoid membrane as a component of the photosystem II (PSII), respectively (Figure 24, Table 1).  
354 These two chloroplastic genes were differentially expressed across nano-, bulk, and ionic forms. A  
355 similar trend was also evident for nLa<sub>2</sub>O<sub>3</sub> and nCuO exposure scenarios. Interestingly, by analyzing  
356 the effects on chloroplast and mitochondrial genomes in *A. thaliana* in terms of copy number, the  
357 effects of nCeO<sub>2</sub> and CeCl<sub>3</sub> exposure were rather limited as compared to the untreated control, which  
358 ~~is in agreement~~ agrees with the limited translocation to the shoots (Pagano et al., 2022). With regard  
359 to proteomic analysis, Majumdar et al. (2015) conducted a quantitative proteomic analysis of kidney  
360 beans (*Phaseous vulgaris* L.) seeds after nCeO<sub>2</sub> exposure and reported that the major seed proteins  
361 associated with nutrient storage (phaseolin) and carbohydrate metabolism (lectins) were significantly

362 reduced by nCeO<sub>2</sub> (62.5-500 mg kg<sup>-1</sup>, 50d exposure) in a dose dependent manner. Interesting, the  
363 plants did not exhibit overt toxicity.

364 In fact, at the physiological level cerium-based nanoparticles generally do not produce phytotoxicity  
365 (Ma et al., 2015; Rui et al., 2014; Lizzi et al., 2020; Rodrigues et al., 2021), though some have  
366 highlighted a positive impact on biomass and on physiological indicators such as chlorophyll and  
367 photosynthesis at selected doses (Rossi et al., 2017; Gui et al., 2017). Another important aspect of  
368 nCeO<sub>2</sub> seems to involve enhance tolerance to saline stress, leading to improved phenotypic and  
369 enzymatic performances and ROS elimination in seeds priming (An et al., 2020; Liu et al., 2021;  
370 Hassanpouraghdam et al., 2022; Chen et al., 2022).

371 Compared to nCeO<sub>2</sub>, nanoscale lanthanum oxide (nLa<sub>2</sub>O<sub>3</sub>) exhibits lower stability, increased ion  
372 dissolution, greater translocation from roots and shoots, all of which seems to lead to higher  
373 phytotoxicity. ~~The limited stability of nLa<sub>2</sub>O<sub>3</sub> has been confirmed by  $\mu$ -XRF analysis performed in~~  
374 ~~*Cucumis sativus* L. (Ma et al., 2015).~~ The limited stability of nLa<sub>2</sub>O<sub>3</sub>, as compared to nCeO<sub>2</sub>, has  
375 been confirmed by  $\mu$ -XRF analysis in *Cucumis sativus* L. through element speciation, dissolution  
376 studies in aqueous solution and *in planta*. After 14d treatment, the nCeO<sub>2</sub> structure in the roots  
377 remains mostly preserved (more than 80%) while pristine nLa<sub>2</sub>O<sub>3</sub> structure was observed at levels  
378 below 10% (Ma et al., 2015).

379 Interestingly, co-contamination with nCeO<sub>2</sub> strongly reduces the uptake of nLa<sub>2</sub>O<sub>3</sub> (Pagano et al.,  
380 2017). The different behaviour of the two ENMs was evident in the transcriptomic profile: only 7 out  
381 of 38 genes were commonly modulated between nCeO<sub>2</sub> and nLa<sub>2</sub>O<sub>3</sub>; these genes were involved in  
382 primary metabolic functions, protein synthesis and stress response (Pagano et al., 2016). Several  
383 publications using different model plants were compared, and the reported effects due to nLa<sub>2</sub>O<sub>3</sub>  
384 exposure in soil include reduction in root and leaf biomass (Ma et al., 2015), decreased transpiration  
385 (Yue et al., 2019), decreased photosynthesis (Xiao et al., 2021) and reduced pigment concentration  
386 (Neves et al., 2019). The decrease in photosynthetic activity is also reflected by altered root  
387 morphology, including root cracking (Xiao et al., 2021) and the presence of apoplastic barriers (Yue

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388 et al., 2019). Interestingly, the adverse effect of nLa<sub>2</sub>O<sub>3</sub> on plant biomass was alleviated under binary  
389 exposure combinations with ENMs such as nCuO and nZnO (Pagano et al., 2017). As ideal case  
390 study, information on RedOx state and potential translocation of lanthanide-based nanoforms within  
391 plant tissues become fundamental in the mechanistic understanding on the physiological and  
392 molecular effects, with regard to application in the agrifood sector.

393

## 394 2.2. *Titanium oxide ENM*

395 Titanium dioxide (nTiO<sub>2</sub>) has been largely studied as a potential environmental and agricultural  
396 contaminant (Servin et al., 2012; 2013). nTiO<sub>2</sub> has demonstrated a high stability, both in anatase and  
397 rutile crystal form (Servin et al., 2012). Translocation of nTiO<sub>2</sub> (0-750 mg kg<sup>-1</sup>, 150d exposure) from  
398 soil to roots and to shoots in *C. sativus* is generally limited, though the two crystal structures were  
399 evident in the leaf trichomes and fruit by μ-XANES spectra (Servin et al., 2013). Ruotolo et al. (2018)  
400 analyzed and reviewed the molecular responses of *A. thaliana* and other model species to nTiO<sub>2</sub> and  
401 reported that exposure triggers an abiotic stress response at the transcriptomic level, involving ROS  
402 detoxification systems, triterpenoid and phenylpropanoid metabolism, and hormone signaling  
403 pathways involving in the response to salicylic acid, jasmonic acid, ethylene, and brassinosteroids.  
404 At the post-transcriptional level, several miRNAs were strongly modulated, including miR395 and  
405 miR399 as key regulators of plant adaptive responses to nutrient starvation (Pagano et al., 2021).  
406 Thus, the ability of nTiO<sub>2</sub> to modulate ROS signaling is particularly effective under abiotic stress  
407 conditions. Here, the presence of this ENMs enhances plant physiological parameters by stimulating  
408 the activation of several defense mechanisms. Several studies (in plants such as *C. sativus*, *S.*  
409 *lycopersicum*, *V. faba*) have shown that in both saline soils and under drought conditions, the addition  
410 of nTiO<sub>2</sub> increases root length, plant biomass, and other parameters such as H<sub>2</sub>O<sub>2</sub> level, antioxidant  
411 activity, sugar content, and chlorophyll amount (Servin et al., 2012; Nasir Kahn, 2016; Abdel Latef  
412 et al., 2018; Mustafa et al., 2021). However, higher concentrations can result in phytotoxicity, likely  
413 due to aggregation and subsequent excessive ROS production (Mattiello et al., 2015; Gohari et al.,

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414 2020). However ROS are “double blade” sword because they can also trigger production of defensive  
415 molecules as shown recently by Castro et al., (2021). Interestingly, when either considering the  
416 utilization of pristine and coated nTiO<sub>2</sub> (hydrophilic or hydrophobic) in carrot (*Daucus carota* L.),  
417 responses observed depended mainly on the nTiO<sub>2</sub> surface coating, concentration and in soil  
418 weathering (Wang et al., 2021a; 2021b). Taproot and leaf fresh biomass and plant height were all  
419 increased with exposure, as well as nutrient uptake (Fe in leaves; Mg in taproots; Ca, Zn, K in roots).  
420 Conversely, sugar and starch contents were negatively affected, compromising the nutritional quality  
421 (Wang et al., 2021b).

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### 424 2.3. *Gold and silver nanoparticles*

425 Similar to nTiO<sub>2</sub>, gold nanoparticles (nAu) are highly stable in plants: nAu remained mostly as Au<sup>0</sup>  
426 within the plant tissues (*Nicotiana tabacum* L. cv. Xanthi nc.-), even if accumulated and translocated  
427 (Sabo-Attwood et al., 2011). Specifically, XANES analyses demonstrated that nAu maintained its  
428 nanoparticle structure without any biotransformation or ionic release. There are no actual uses for  
429 gold nanoparticles and plants, it just used as a tool to study NP-plant interactions. nAu levels in  
430 biosolids would ever be high enough to be considered phytotoxic. It is known that nAu toxicity  
431 depends on concentration, particle size and shape: nAu with a smaller particle size (3.5nm,  
432 concentration of 48 mg L<sup>-1</sup>) were evenly biodistributed across the plant in comparison with the  
433 18.5nm nAu (in a concentration of 76 mg L<sup>-1</sup>), even leading to the formation of necrotic leaf lesions  
434 and plant death after 30 days (Sabo-Attwood et al., 2011). Other studies have shown that nAu  
435 exposure improved radical scavenging and antioxidant enzymatic activities and modulated miRNA  
436 expression implicated plant abiotic stress response (miR398, miR408). In particular, the regulation  
437 of superoxide dismutase (SOD) led to an increased ROS scavenging activity, root elongation,  
438 seedling growth, and seed yield (Arora et al. 2012; Kumar et al., 2013; Siddiqi & Husen, 2017).

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439 Given the widespread commercial utilization and environmental relevance (*e.g.*, wastewater  
440 treatment; fertilization) of silver nanoformulations (nAg), the effect on plant species has been a topic  
441 of robust study. Stegemeier et al. (2015) analyzed the nAg and nAg<sub>2</sub>S speciation in *Medicago sativa*  
442 L., demonstrating that nAg accumulates in the root elongation area but that nAg<sub>2</sub>S remains adhered  
443 to the root surface; Ag ions accumulate more uniformly throughout the root tissues. Notably, the Ag  
444 accumulation in the root apoplast was determined by XRF. The presence of nAg in root cell walls  
445 demonstrated the uptake of partially dissolved nAg and translocation along the apoplast. Larue et al.  
446 (2014) localized and determined nAg speciation in *L. sativa* after foliar spray treatment through  $\mu$ -  
447 XRF and  $\mu$ -XAS techniques; the authors reported that nAg was able to cross the foliar cuticle,  
448 penetrating in the leaf tissue through the stomata. Moreover, nAg biotransformed through oxidation  
449 and complexation with thiol-containing molecules such as glutathione (GSH). These findings  
450 correlated well with the transcriptomics analyses of *A. thaliana* exposed to different types of nAg:  
451 plant response included defensin-like proteins, plant thionin,  $\beta$ -glucosidases, cytochrome P450  
452 proteins, and glutathione-S-transferase (GST) members (Kaveh et al., 2013). Although some studies  
453 point out that the morphological and physiological effects of nAg exposure were strictly dependent  
454 on particle size and concentration and that sublethal concentrations may have also beneficial effects  
455 (Wang et al., 2013; Syu et al., 2014), most of reports demonstrated reduced root elongation and shoot  
456 biomass, together with decreased levels of chlorophyll, pigments, micronutrients, and increased level  
457 of ROS and activity of enzymes involved in the oxidative stress response (Yin et al., 2011; Zuverza-  
458 Mena et al., 2016; Yang et al., 2018; Lahuta et al., 2022).

459

#### 460 2.4. *Iron-based ENMs*

461 Iron-based nanomaterials, including iron oxides (nFeOx) and zero valent iron (nZVI), have been  
462 investigated in plant systems and the reports highlight two major routes of entry: i) a reductive and  
463 proton-promoted process able to modify the structure of the ENM or ii) through the secretion of plant  
464 transporters (*e.g.*, phytosiderophores) with a high affinity for Fe (III) (Morrissey & Guerinot, 2009).

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465 Dwivedi et al. (2018) investigated nZVI exposure in *C. sativus* and reported that transformed nZVI  
466 was stored in the root cell membrane and vacuoles of the leaf parenchyma. XAS identified ferric  
467 citrate and iron (oxyhydr)oxides as the main transformation products in roots and shoots, albeit in  
468 different proportions. The major pathways of nZVI biotransformation involve interaction with low  
469 molecular weight organic acid ligands and on the dissolution/precipitation of the mineral products.  
470 Transcriptional analyses performed on H<sup>+</sup>-ATPase genes (*CsHA1*, *CsHA2*) showed an upregulation  
471 of these genes upon nZVI exposure (and relative root acidification), indicating that the plant-  
472 promoted transformation of nZVI can be driven by protons released by the roots.

473 A separate study investigated the effects of nFe<sub>2</sub>O<sub>3</sub> and nFe<sub>3</sub>O<sub>4</sub> on *A. thaliana*, highlighting  
474 differences in the response between nanoparticle forms and metal salts through a nanoscale-specific  
475 response pathway involving energy production and oxidative stress. The differential response was  
476 ascribed to the ENM and the metal salt dissolution rates and the toxicity of the metal ion, which is  
477 more compatible with biotransformation processes in the plant tissues. Importantly, specific effects  
478 on plastid and mitochondrial genomes were evident, with nFeOx causing a 1- to 3-fold increase in  
479 ptDNA and mtDNA copy numbers depending on the stability of the nanoform utilized (Pagano et al.,  
480 2022).

481 Given their widespread application in soil and water remediation, a primary concern with iron  
482 nanoparticles is a potential toxicity from excessive accumulation in the environment. However,  
483 several studies have shown that plant exposure to this type of nanoparticle does not result in  
484 phytotoxicity. For example, Dwivedi et al. (2018) evaluated the potential environmental impact of  
485 nZVI on *C. sativus* in soil and in hydroponic culture, and reported no instances of reduced plant  
486 biomass even at the highest doses (from 250 to 1000 mg L<sup>-1</sup>) and for *O. sativa*, the low doses (50-500  
487 mg L<sup>-1</sup>) of nZVI and nFe<sub>3</sub>O<sub>4</sub> improved plant growth (Li et al., 2021). The use of this nanomaterial as  
488 a soil conditioner for remediation of metal-contaminated soils is confirmed by the demonstration of  
489 improved plant growth in Cd-contaminated soils (Rizwan et al., 2019; Manzoor et al., 2021);

490 mechanistically, this involves limiting cadmium translocation and the promotion of antioxidant  
491 activity.

492 In summary, the extent and the degree of biotransformation of nZVI, which consists in the  
493 biochemical alteration of chemical compounds within a living tissue, are reflected in the physico-  
494 chemical properties, macromolecular interaction, and biologically mediated pathways observed.

495

#### 496 2.5. Zinc-based ENMs

497 Zinc-based nanomaterials have been applied to plants to increase food safety, promote food  
498 production and enhance sustainability by reducing oxidative stress symptoms induced by abiotic  
499 stressors (Faizan et al., 2021). nZnO is characterized by a low stability, and a high dissolution rate  
500 (Lv et al., 2021). Hernandez-Viezcas et al. (2013) exposed *Glycine max* L. to nZnO (500 mg kg<sup>-1</sup>,  
501 48d exposure):  $\mu$ -XRF analysis showed no detectable ZnO NPs within the tissues, while  $\mu$ -XANES  
502 data showed O-bound Zn in a form resembling Zn citrate. Lv et al. (2015) studied the effects of nZnO  
503 in *Z. mays* L. and used  $\mu$ -XANES to demonstrate that the majority of accumulated Zn was derived  
504 from Zn<sup>2+</sup> released from the nanoparticles and was accumulated mainly as Zn phosphate in epidermis,  
505 cortex, and root tip cells. The results were correlated to transcriptomic analyses in which gene  
506 ontology (GO) performed in nZnO-exposed *A. thaliana* revealed significant commonalities with the  
507 response to Zn<sup>2+</sup> ions, particularly with proteins involved in metal binding, transport, metal  
508 homeostasis and detoxification. This suggests that Zn ion release by nZnO is a key in mediating the  
509 overall effect on plant species (Landa et al., 2015). These findings have been extended to other  
510 species, such as *C. pepo* L.; here nZnO treatment was shown to modulate genes that encode for  
511 transporters of heavy metals, cellular response to abiotic stress, decreased chlorophyll production,  
512 and induction of secondary metabolite biosynthesis (Pagano et al., 2017).

513 In recent years other forms of Zn-based nanomaterials have been tested for a potential plant  
514 remediation purpose, such as ZnS QDs (Imperiale et al., 2022). An analysis of the effect of ZnS QDs  
515 and ionic Zn exposure on mitochondrial and plastid genome copy number demonstrates that both

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516 increase by 1 to 3-fold), but that ZnS QDs dissolution alone does not explain the phenomenon; this  
517 suggests that ZnS QDs biotransformation may occur within the plant tissues and organs to a form  
518 more similar to ionic than nanoscale Zn (Pagano et al., 2022). Zinc-based nanomaterials have also  
519 shown interesting properties as nanofertilizers, including mitigating abiotic and biotic stress (*e.g.*, salt  
520 stress, infections), regulating micronutrient uptake, improving water use efficiency, and promoting  
521 detoxification of heavy metals (Akhtar et al., 2021; Zafar et al., 2022). Under drought conditions, the  
522 nZnO (5 mg kg<sup>-1</sup>) significantly increased grain yield in sorghum (*Sorghum vulgare* Moench) and fruit  
523 yield in eggplant (*Solanum melongena* L.), respectively by 22-183% and 12-23% (Dimkpa et al.,  
524 2019; Semida et al., 2021).

525

## 526 2.6. -Copper oxide

527 Copper oxide nanomaterials (nCuO) are among the most utilized ENMs with plants, including use as  
528 a nanopesticide or nanofertilizer (Elmer et al., 2018; Lowry et al., 2019; Xu et al., 2022). ~~Copper~~  
529 ~~oxide nanomaterials (nCuO) are one of the most utilized ENMs with plants, including use as a~~  
530 ~~nanofertilizer (Elmer et al., 2018; Lowry et al., 2019).~~ nCuO dissolution within the plant tissues has  
531 been demonstrated (in *C. pepo*), and this was shown to depend not only on uptake, and translocation,  
532 but also on the interaction with important biomolecules (Tamez et al., 2019; Marmioli et al., 2021).  
533 EXAFS (Marmioli et al., 2021) demonstrated that the local Cu environment in the higher shells  
534 shows small differences between roots and flowers. A second Cu-O shell path was present in both  
535 flowers and roots; a Cu-Cu bond was also observed in ~~roots, but~~ roots but was not observed in flowers.  
536 A full transcriptomics analysis by RNAseq was performed to highlight the differential responses  
537 between nano-, bulk and ionic forms in roots, leaves and pollen (Marmioli et al., 2021). The results  
538 highlighted the nano-specificity of the responses; the modulated genes (significantly up- or down-  
539 regulated genes) observed were more significant in the roots and decreased with translocation to  
540 leaves and pollen. However, the portion of the response common to the three Cu forms tested was  
541 shown to increase following the translocation from roots to shoots (Marmioli et al., 2021). A

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542 characterization of the main steps and implications involved in this phenomenon, as well as some  
543 relevant biomarkers observed in different plant species, is presented in Figure 2+ (details reported in  
544 Table 1).

545 Additional data was presented by Servin et al. (2017a), who studied nCuO weathering in *Lactuca*  
546 *sativa* L.: lettuce was exposed to unweathered and 70d-weathered nCuO, and corresponding bulk and  
547 ionic form (0–400 mg kg<sup>-1</sup>) for 70 d in soil. To assess nCuO trophic transfer, leaves were fed to  
548 crickets (*Acheta domestica* L.) as primary consumer, followed by insect feeding to lizards (*Anolis*  
549 *carolinensis* L.) as secondary consumer, in both cases for 15d. The authors used μ-XANES to show  
550 that Cu(II) was reduced to Cu(I) within the plant roots, and used a transcriptional analysis of to show  
551 that several biomarkers, including *CCH* and *COPT5*, which encodes for a copper chaperon and a  
552 copper ion transporter, respectively, were significantly decreased by weathering.

553 In spite of being widely used, results regarding the physiological effects upon nCuO exposure are  
554 rather discordant. For example, Deng et al. (2022a) reported that, unlike the bulk counterpart, nCuO  
555 (0-600 mg kg<sup>-1</sup> of soil) does not produce toxicity in rice (*O. sativa*), but rather improves the supply  
556 of essential elements, including increasing content of sugar and starch, as well as overall yield.

557 The grain of weedy and cultivated rice were differentially impacted by nCuO, bulk or ionic forms,  
558 showing also a cultivar-specific and concentration-dependent response. Cu translocation directly  
559 influenced plant yield, sugar production, starch content, protein content, and expression of auxin  
560 associated genes in grain (Deng et al., 2022b). Analyzing the effect of citric acid (CA) coated copper  
561 oxide (CA-nCuO) and its application (foliar spray or soil exposure) on the growth and physiology of  
562 soybean (*Glycine max* L.), nCuO appeared to be more accessible for plant uptake, as compared to  
563 CA-nCuO, decreasing the protein content, and inhibiting plant growth. CA reduced CuO NPs  
564 toxicity, demonstrating that surface modification may change the toxic properties of NPs. (Deng et  
565 al., 2022c).

566 Treatment of *Lactuca sativa* L. with nCuO significantly increased biomass as compared to CuO  
567 microparticles (Wang et al., 2019). In addition, plants can benefit from nCuO treatment through

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568 enhanced defensive pathways, and through direct antimicrobial and antifungal activities (Elmer et al.,  
569 2018). Similarly, *L. sativa* treatment with nCuO significantly increased biomass relative to CuO  
570 microparticles (Wang et al., 2019). In addition, nCuO can benefit plants through enhanced defensive  
571 pathways, as well as through direct antimicrobial and antifungal activities (Elmer et al., 2018). For  
572 example, exposure of nCuO to *Solanum lycopersicum* increased root and stem length, leaf number,  
573 and chlorophyll content, and also inhibited the mycelial growth of *Fusarium oxysporum* sp.  
574 *Lycopersici* (Lopez-Lima et al., 2021). Conversely, some authors report toxic and inhibitory effects  
575 on the growth in plants such as lettuce (*Lactuca sativa* L., 0-1000 mg L<sup>-1</sup>, 5-15d exposure by foliar  
576 spray), turnip (*Brassica rapa* L., 50-500 mg L<sup>-1</sup>, 14d exposure), and wheat (*Triticum aestivum* L., 50  
577 mg kg<sup>-1</sup> in sand, 1-14d exposure) upon nCuO treatment. The toxic effects are largely ascribed to the  
578 redox reactivity and ROS generation of the nanoparticle form (Dimkpa et al., 2012; Chung et al.,  
579 2019; Xiong et al., 2020). Others have reported no significant impact at the physiological level  
580 (Servin et al., 2017a; Tamez et al., 2019; Marmioli et al., 2021; Roubeau Dumont et al., 2022), which  
581 highlights the importance of the experimental variables and design, including dose, particle  
582 properties, exposure conditions and endpoints.

583

### 584 2.7. Quantum dots

585 Cadmium-based nanomaterials, and cadmium sulfide quantum dots (CdS QDs) in particular, have  
586 been used as a model material to elucidate physiological mechanisms and molecular pathways  
587 involved in the response plant response to exposure (Marmioli et al., 2014; Imperiale et al., 2022).  
588 A Systems biology approach gave a complete picture of the targets in both model (*A. thaliana*) and  
589 crop (*C. pepo*) species (Marmioli et al., 2014; Marmioli et al., 2015; Pagano et al., 2017; Gallo et  
590 al., 2021; Marmioli et al., 2020; Pagano et al., 2022). In *A. thaliana*, CdS QDs tolerant mutants were  
591 used to establish *in vitro* inhibition concentrations for growth (80 mg L<sup>-1</sup>) in an attempt to elucidate  
592 the mechanisms involved in the plant response; the results largely implicated metabolic functions and  
593 chloroplast energy production as sensitive targets (Marmioli et al., 2014). The results demonstrate

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594 that CdS QDs and ionic Cd were exploiting different pathways in the plant, highlighting that the  
595 tolerance to CdS QDs did not ~~overlapped~~overlap with the tolerance to CdSO<sub>4</sub>. Conversely, Cd  
596 sensitive mutants of Arabidopsis (Howden & Cobbett, 1992) that were exposed to CdS QDs did not  
597 exhibit differences in growth as compared to the wild type line (Marmioli et al., 2014). A  
598 transcriptomic analysis and proteomic comparison between wild type and tolerant mutants  
599 highlighted that only a few genes were commonly modulated upon ionic Cd and CdS QDs treatment  
600 (Marmioli et al., 2015, Gallo et al., 2021). Marmioli et al., (2020) used EXAFS to investigate the  
601 cadmium environment in planta and showed that the spectra were compatible with a mixed O/S  
602 coordination; while Cd–S distances did not show relevant variations, Cd–O distances varied in  
603 samples grown with QDs compared with those grown with CdSO<sub>4</sub>. The number of Cd–S bonds in  
604 plants grown with QDs was higher than Cd–O bonds. This EXAFS analysis demonstrated that CdS  
605 QDs were biotransformed after uptake: the QD original structure was modified but not completely  
606 absent within the plant cell, and Cd atoms were not released as Cd ions. Interestingly, CdS QDs  
607 showed a relatively high stability; once accumulated by the plant, the QD may go through different  
608 stages in the response pathways: i) exposure: explained by the different genetic mechanisms behind  
609 the physiological/molecular response between the wild type and tolerant phenotypes; ii)  
610 reactivity/biotransformation: explained by a transition phase in which the structure of CdS QDs is  
611 modified to decrease particle reactivity, and this can be detected by XANES and EXAFS analyses;  
612 iii) effects/detoxification: transcriptomic, proteomic and metabolomic response related to the  
613 physico-chemical forms after QDs biotransformation. Additionally, the effects on organelle genomes  
614 (ptDNA and mtDNA) demonstrate how QDs biotransformation may modify the genomes  
615 stoichiometry or sub-stoichiometry, likely through potential morpho-functional adaptive response  
616 triggered by modifications in the bioenergetic redox balance, or a reduction of photosynthesis or  
617 cellular respiration rates after QD exposure (Pagano et al., 2022).

618 Similarly to what was observed in *A. thaliana*, CdS QDs induced analogous effects other plant species  
619 of agricultural interest: Pagano et al. (2017) analyzed the effects of the CdS QDs in a context of

620 ENMs binary co-contamination, highlighting a similar response as in *A. thaliana*; specific and  
621 common biomarkers were involved between CdS QDs and other the ENMs tested (nCeO<sub>2</sub>, nLa<sub>2</sub>O<sub>3</sub>,  
622 nCuO, nZnO). Majumdar et al. (2019) investigated the effect of differently functionalized CdS QDs  
623 in *G. max*; the authors used proteomic and metabolomic endpoints to demonstrate how the  
624 transmembrane proteins involved uptake and related genes including *NRAMP6* and *HMA8* were  
625 differently regulated in CdS QDs and ion treated plants. In addition, ATP-dependent ion transporters  
626 in the membranes presented feedback mechanisms in the soybean roots to restrict the uptake of CdS  
627 QDs and simultaneously to alter the mineral acquisition. Moreover, CdS QDs altered major metabolic  
628 functions, including glutathione metabolism, the tricarboxylic acid cycle, glycolysis, fatty acid  
629 oxidation and phenylpropanoid and amino acids biosynthesis. Physiologically, CdS QDs, induced  
630 oxidative stress, decreased biomass, reduced chlorophyll and carotenoids content, and damaged  
631 primary roots (Majumdar et al., 2019; Pagano et al., 2022).

632  
633

### 634 3. *Biotransformation as a perspective to comprehend ENM response in plant*

635 ENMs have been rather extensively tested in recent years, with data indicating that several physico-  
636 chemical parameters are fundamental to explaining their behaviour during exposure, including  
637 composition, stability, surface charge, and functionalization. These ENM properties become  
638 biologically relevant and mediate subsequent biotransformation processes, including: i) the  
639 possibility to be translocated within organs, tissues, and cells; ii) the ability to interact with the  
640 biologically active environment within the plant (*e.g.*, phospholipids, nucleic acids, proteins,  
641 secondary metabolites, reactive oxygen species); iii) the dissolution rate and the consequent ion  
642 release. Importantly, to fully comprehend the plant response to ENMs exposure, one must consider  
643 the biologically modified ENMs forms that are indicative of the highly complex interactions between  
644 plants and ENMs interaction. Integration of the information from physiological and molecular  
645 analyses with physical evidence (*e.g.*, types and number of atoms surrounding the ENM, radial

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646 distance between atoms of the interactors and atoms constituting the ENM; ENM crystal structure)  
647 obtained through high energy X-ray spectroscopy platforms such as synchrotron-based techniques  
648 will enable a more realistic, mechanistic, and systems-level picture of ~~of~~-plant response to ENM  
649 exposure.

650 This review describes some of the primary biological constraints that determine ENM  
651 biotransformation in plants (Figure 24, Table 2). For ENMs characterized by high stability, such as  
652 nCeO<sub>2</sub>, nTiO<sub>2</sub> or nAu, limited dissolution and translocation has been observed, even considering  
653 differences determined by structure and atomic properties related to the redox state (*e.g.*, the redox  
654 state of Ce). Conversely, ENMs with higher dissolution such as Fe- or Zn-based ENMs, nCuO, nAg  
655 or nLa<sub>2</sub>O<sub>3</sub>, exhibit greater translocation rates, likely involving a dynamic process of particle  
656 interaction with the plant biomolecules that increase ENM solubility and bioavailability, as  
657 exemplified with nCuO (Marmiroli et al., 2021).

658 The importance of in planta ENM biotransformation is corroborated indirectly at molecular level by  
659 “omic” analyses that can describe the effects on the plant at genetic and epigenetic level (including  
660 genome stability) by measuring transcriptional modulation, protein abundance and metabolite  
661 synthesis, as well as on physiological (phenotypical) level by observing the plant redox state, ROS  
662 production, photosynthetic activity, and cellular respiration rate in response to stress (Marmiroli et  
663 al., 2020; Gallo et al., 2021). The direct measurement of changes upon ENM biotransformation within  
664 the plant tissues by synchrotron-based techniques ( $\mu$ -XRF,  $\mu$ -XANES, and XAS) provide critical  
665 information in terms of distribution, atomic redox state, and atomic local structure, and add critical  
666 knowledge necessary to understand the ENM-plant interactions. This information is highly relevant  
667 with regard to potential applicability: ENMs can interact with sensitive ecosystem components within  
668 trophic food chains, affect microbial populations in soil, enter into the plant and where they can be  
669 translocated to different tissues and organs, including the edible tissues or organs (Holden et al., 2013;  
670 Liu et al., 2015). Biotransformation can occur at each step within these processes, modifying and/or  
671 amplifying ENM effects at organism level. These interactions from the level of ecosystem, organism,

672 ~~tissue, cell, and organelles become key factors when applying “ENM biotransformation” as a concept~~  
673 ~~for a safer design, when considering applications for agriculture and food production, and for~~  
674 ~~minimizing the adverse biological impact (Burello & Worth, 2015; Pagano et al., 2018; Lowry et al.,~~  
675 ~~2019; Kah et al., 2019; Zulfiqar et al., 2019; Ma et al., 2020; Hameed et al., 2022; Xu et al., 2022)This~~  
676 ~~information is highly relevant with regard to applications: ENMs can clearly interact with sensitive~~  
677 ~~ecosystem components and within the trophic food chains, alter microbial populations in soil, enter~~  
678 ~~in plants and be translocated to different tissues and organs, including edible tissues (Holden et al.,~~  
679 ~~2013; Liu et al., 2015). Biotransformation can occur at each step of these processes, modifying and/or~~  
680 ~~amplifying the effects ENM effects at the level organisms. These interactions at the level of~~  
681 ~~ecosystem, organism, tissue, cell, and organelles become key factors in applying “ENM~~  
682 ~~biotransformation” as a concept for safer design when considering applications for agriculture and~~  
683 ~~food production, for minimizing the adverse biological effects (Burello & Worth, 2015; Pagano et~~  
684 ~~al., 2018; Lowry et al., 2019; Kah et al., 2019; Zulfiqar et al., 2019; Ma et al., 2020; Hameed et al.,~~  
685 ~~2022).~~

#### 688 *Author contributions*

689 Conceptualization: LP, MM; original draft preparation: LP, MM, RR; review and editing: JCW, NM.

690 All authors revised and agreed on the final version of the manuscript.

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699 The authors declare no competing financial interests.

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1215 **Figure captions and Tables**

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1218 Figure 1. Schematic representation of principal effects emerging after biotransformation of ENMs in  
1219 soil, on foliar surface and within the plant tissues. ENMs in soil, due to particle and soil physico-  
1220 chemical interactions, may remain unmodified, or may undergo to potential dissolution (with ions  
1221 release), or undergo homo-/hetero-aggregation, which may highly influence particle stability and  
1222 potential bioavailability (and consequently their uptake). On the foliar surface, temperature and light  
1223 may also affect particle stability before uptake into the leaf tissues. Once within the plant, ENMs  
1224 pristine, or modified, can interact with several biomolecule types (peptides, sugars, lipids, nucleic  
1225 acids, secondary metabolites) leading to phenomena such as coating, enzymatic degradation,  
1226 chelation or functionalization, which may influence the biotransformed particle at level of  
1227 translocation, storage or reactivity. These parameters may also influence the interaction within the  
1228 plant cell, triggering differential responses (e.g., toxicity, oxidative stress, ROS production), which  
1229 may be indirectly measured by physiological and molecular assays, but directly observed through  
1230 physical strategies, including synchrotron-based methods.

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1232 Figure 24. Principal mechanisms effects of ENM biotransformation in plant and relevant biomarkers  
1233 observed in different plant species from model organisms (*A. thaliana*) to crops (*C. pepo*; *S.*  
1234 *lycopersicum*; *G. max*) and different tissues (roots, leaves and flowers/pollen). Relevant ENM  
1235 parameters such as size, stability, dissolution may influence the translocation from roots to shoots.  
1236 Potential biotransformation mechanisms that may occur within plant tissues are also reported:  
1237 enzymatic degradation, protein functionalization, functionalization at the level of cytoplasm and  
1238 organelles (organic acids, thiol-containing compounds, aminoacids, sugars, secondary metabolites).

1239 In this scenario, chloroplast become not only a in important actor in the energy production but also  
 1240 one of the key targets and main regulators involved in the ENM exposure and response. Details on  
 1241 the biomarkers generated are reported in Table 1.

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1252 Table 1. Genes as potential biomarkers of exposure/effect observed in roots, leaves and pollen, in  
 1253 different plant species (reported in Figure 2). Genes reported cannot be considered only as modulated  
 1254 in the different plant organs by the different type of ENMs, but also they showed a nano-specificity  
 1255 during the ENM response. It is also important to observe how, depending on the ENM type,  
 1256 biotransformation, and as indirect consequence, the transcriptomic response can be convergent  
 1257 between different forms (nano, bulk, ion) of the same element (see Figure 2).~~Table 1. Gene list of~~  
 1258 ~~potential biomarkers observed in roots, leaves and pollen, in different plant species (reported in Figure~~  
 1259 ~~1):~~

flower & pollen					
plant	gene	function	pathway	ENM	reference
<i>Cucurbita pepo L.</i>	Cp4.1LG00g07430	Beta-galactosidase	primary metabolism		
	Cp4.1LG13g06380	Pectinesterase	primary metabolism		
	Cp4.1LG12g04750	Phosphatidylinositol 3-/4-kinase family protein	primary metabolism	nCuO	Marmioli <i>et al.</i> , 2021
	Cp4.1LG03g02560	Delta-1-pyrroline-5-carboxylate synthetase	primary metabolism		
	Cp4.1LG02g07240	Leucine-rich repeat family protein	signaling, stress response		

Cp4.1LG02g12750	Protein EFR3 like	signaling
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leaf					
plant	gene	function	pathway	ENM	reference
<i>Cucurbita pepo</i> L.	Cp4.1LG01(*)	PSBN, photosystem II reaction center protein N	chloroplast electron transport	nCeO2 nLa2O3	Pagano <i>et al.</i> , 2016 Pagano <i>et al.</i> , 2017
	Cp4.1LG00(*)	PetL, component of Cytochrome b6f	chloroplast electron transport	nCuO nZnO CdS QD	
<i>Solanum lycopersicum</i> L.	Solyc09g074540	PetL, component of Cytochrome b6f	chloroplast electron transport	nCeO2 nLa2O3 nCuO	Pagano <i>et al.</i> , 2016
<i>Glicine max</i> L.	Glyma12g36130	PetL, component of Cytochrome b6f	chloroplast electron transport	CdS QD	Majumdar <i>et al.</i> , 2019
<i>Arabidopsi thaliana</i> L.	AtCg00590	PetL, component of Cytochrome b6f	chloroplast electron transport	CdS QD	Marmioli <i>et al.</i> , 2014
	AtCg00270	PSBN, photosystem II reaction center protein N	chloroplast electron transport		

root					
plant	gene	function	pathway	ENM	reference
<i>Cucurbita pepo</i> L.	Cp4.1LG16g08630	BIP3, Heat shock protein 70 family protein	protein folding, stress response	nCeO2 nLa2O3 nCuO nZnO CdS QD	Pagano <i>et al.</i> , 2016 Pagano <i>et al.</i> , 2017
	Cp4.1LG00g00090	GPT2, glucose-6-phosphate/phosphate translocator	primary metabolism		
	Cp4.1LG05g08050	RPS12, ribosomal protein S12A	protein synthesis		
	Cp4.1LG05g11200	PLP2, phospholipase	biotic/abiotic stress response		
<i>Solanum lycopersicum</i> L.	Solyc08g006150	ChaC-like family protein	glutathione degradation	nCeO2 nLa2O3 nCuO	Pagano <i>et al.</i> , 2016
	Solyc03g081240	PRR5, pseudo-response regulator 5	biotic/abiotic stress response		
	Solyc10g005080	LHY1, Homeodomain-like superfamily protein	stress response		
<i>Glicine max</i> L.	Glyma19g45030	LHY1, Homeodomain-like superfamily protein	stress response	CdS QD	Majumdar <i>et al.</i> , 2019
	Glyma15g06800	PR1, pathogenesis-related gene 1	biotic/abiotic stress response		

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Table 2. Principal evidence of the ENM biotransformation in plant observed by physiological, molecular and synchrotron-based analyses.

ENM	plant	physiological response	molecular response	techniques	biotransformation	reference
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nCeO2	<i>Cucumis sativus L.</i>	nCeO2 exposure had no significant effects on the biomass production under both the +P and -P conditions. However, the uptake of Ce in the plants is different under the two conditions	-	TEM XANES NEXAFS	high stability	<i>Rui et al., 2015</i>
	<i>Lactuca sativa L.</i> <i>Cucurbita pepo L.</i> <i>Zea mays L.</i> <i>Glycine max L.</i>	biomass in the agricultural soil amended with biochar 600°C was largely unaffected	-	SEM μ-XRF μ-XANES	modified redox state, from Ce(IV) to Ce(III) low translocation from roots to shoots	<i>Servin et al., 2017</i>
nCeO2 nZnO	<i>Glycine max L.</i>	-	-	μ-XRF μ-XANES		<i>Hernandez-Viezcas et al., 2013</i>
nCeO2 nLa2O3	<i>Cucumis sativus L.</i>	nCeO2 had no phytotoxicity to cucumber at all tested concentrations, while nLa2O3 showed significant inhibition on root elongation, shoot elongation, root biomass, and shoot biomass, as well as induced more reactive oxygen species and cell death in roots	-	μ-XRF XAS	higher dissolution compared to nCeO2 moderate translocation from roots to shoots	<i>Ma et al., 2014</i>
nTiO2	<i>Cucumis sativus L.</i>	at all concentrations, nTiO2 significantly increased root length (average >300%)	-	μ-XRF μ-XANES	high stability	<i>Servin et al., 2012</i>
	<i>Cucumis sativus L.</i>	In nTiO2 treated plants, the chlorophyll content in leaves increased as the external concentration of NPs increased. nTiO2 treatments increased CAT activity in leaves.	-	μ-XRF μ-XANES FTIR	low translocation from roots to shoots	<i>Servin et al., 2013</i>
nAu	<i>Nicotiana tabacum L.</i>	leaf necrosis was observed after 14 days of exposure to 3.5 nm nAu	-	μ-XRF	high stability no changes in Au valence	<i>Sabo-Attwood et al., 2012</i>
nAg	<i>Lolium multiflorum L.</i>	nAg and ionic silver significantly reduced growth, resulting in shorter shoots and roots and lower biomass. The growth inhibition from nAg was stronger than that from AgNO3. Higher concentrations of AgNPs caused broken epidermis and rootcap. Cell structures were unaltered in AgNO3 treated roots.	-	μ-XRF XANES	low stability high translocation from roots to shoots	<i>Yin et al., 2011</i>
	<i>Lactuca sativa L.</i> <i>Cucurbita pepo L.</i> <i>Zea mays L.</i> <i>Glycine max L.</i>	fresh foliar biomass was unchanged. Chlorophyll a, chlorophyll b, carotenoid and pheophytin contents were not affect	-	SEM μ-XRF XANES		<i>Larue et al., 2013</i>
	<i>Medicago sativa L.</i>	-	-	TEM XRF		<i>Stegemeier et al., 2015</i>
nZVI	<i>Cucumis sativus L.</i>	nZVI treatments did not affect the biomass of plants in hydroponic or soil systems. Only nZVI treated plant shoots grown under hydroponic conditions exhibited increased biomass (15%). Chlorosis observed in the leaves of the control plants but not in the plants treated with nZVI	ATPase isoforms increased their expression in the roots of plant exposed to nZVI.	EXAFS	low stability limited translocation from roots to shoots modified particle structure	<i>Dwivedi et al., 2018</i>

Table 2 continue in the next page...

ENM	plant	physiological response	molecular response	techniques	biotransformation	reference
nZnO	<i>Zea Mays L.</i>	-	-	μ-XRF XANES	low stability	<i>Ly et al., 2015</i>

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	<i>Zea Mays L.</i>	By the 7th day, the treatment of 9 nm nZnO and ZnSO <sub>4</sub> significantly reduced the dry weight of roots by 44% and 58% respectively, compared to the unexposed control plants. In general, ZnSO <sub>4</sub> treatment had the greatest effect on root biomass, followed by 9 nm nZnO and finally 40 nm nZnO	-	μ-XRF	high translocation from roots to shoots	<i>Ly et al., 2021</i>
nCuO	<i>Nicotiana tabacum L.</i>	When exposed to equivalent weight of Cu, nCu <sub>2</sub> O exhibited higher toxicity than nCuO, implying that the transformation may elevate the toxicity upon nCuO exposure	-	TEM XANES		<i>Dai et al., 2019</i>
	<i>Lactuca sativa L.</i>	Cu exposure had limited impacts on lettuce biomass. For the unweathered exposures, only the root biomass of NP-exposed plants was less than in bulk treatment; no other tissue-specific differences were evident. In the W exposure, the total biomass ranged from 8.2 g (W NP) to 9.5 g (unexposed control); nCuO and ion-treated plant biomass was significantly less than the unexposed controls. With regard to individual tissues in the W exposure, there were no differences of significance in the root biomass.	The expression level of nine genes involved in Cu transport shows that the mechanisms of nCuO and bulk CuO response-accumulation are different from ionic Cu	μ-XRF XANES	low stability high translocation from roots to shoots consistent with an increased ion release modified redox state, from Cu(II) to Cu (I)	<i>Servin et al., 2017</i>
	<i>Cucurbita pepo L.</i>	no impact on zucchini biomass, photosynthetic activity or cellular respiration.	RNA-seq analyses on vegetative and reproductive tissues highlighted common and nanoscale-specific components of the response. Mitochondrial and chloroplast functions were uniquely modulated in response to ENM exposure as compared with bulk and salt forms	μ-XRF XANES EXAFS		<i>Marmioli et al., 2021</i>
CdS QD	<i>Arabidopsis thaliana L.</i>	treatment with CdS QDs caused a slight stress that increased the biomass in the mutants, but not in the wt, while CdSO <sub>4</sub> caused modest phytotoxicity to both the wt and mutants	-	EXAFS	high stability limited ion release high translocation modification in bonds distance	<i>Marmioli et al., 2020</i>

# 1 **Nanomaterials biotransformation: In planta mechanisms of action**

2

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15

## 16 **Abstract**

17 Research on engineered nanomaterials (ENMs) exposure has continued to expand rapidly, with a  
18 focus on uncovering the underlying mechanisms. The EU largely limits the number and the type of  
19 organisms that can be used for experimental testing through the 3R normative. There are different  
20 routes through which ENMs can enter the soil-plant system: this includes the agricultural application  
21 of sewage sludges, and the distribution of nano-enabled agrochemicals. However, a thorough  
22 understanding of the physiological and molecular implications of ENMs dispersion and chronic low-  
23 dose exposure remains elusive, thus requiring new evidence and a more mechanistic overview of  
24 pathways and major effectors involved in plants. Plants can offer a reliable alternative to conventional  
25 model systems to elucidate the concept of ENM biotransformation within tissues and organs, as a  
26 crucial step in understanding the mechanisms of ENM-organism interaction. To facilitate the



27 understanding of the physico-chemical forms involved in plant response, synchrotron-based  
28 techniques have added new potential perspectives in studying the interactions between ENMs and  
29 biota. These techniques are providing new insights on the interactions between ENMs and  
30 biomolecules. The present review discusses the principal outcomes for ENMs after intake by plants,  
31 including possible routes of biotransformation which make their final fate less uncertain, and  
32 therefore require further investigation.

33

34 **Keywords:** biotransformation, plant, nanomaterials, synchrotron-based analyses, molecular response

35

### 36 **Highlights**

- 37 • Biotransformation is a fundamental phenomenon for understanding ENM-organism response
- 38 mechanisms
- 39 • Synchrotron-based methodological analyses are critical for investigating ENM
- 40 biotransformation
- 41 • Biotransformation of ENMs may have positive or negative effects when considering the agri-
- 42 food application

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## 45 **1. Engineered nanomaterial (ENM) biotransformation**

### 46 *1.1. ENMs: from exposure to biotransformation*

47 Although global food production has generally increased over time, the distribution has been far from  
48 equitable, with more than 820 million people having insufficient food and many more consuming  
49 low-quality diets leading directly to micronutrient deficiencies (Willett et al 2019; Zhong et al., 2020).  
50 In the past 20 years, and particularly in the past decade, engineered nanomaterials (ENMs) have seen  
51 dramatically increasing use and had an equally significant impact on ecosystems services and human  
52 society. As such, studies focused on the implications associated with their use are critical. In fact, it

53 is known that ENMs exert important, but not completely understood, effects on biota; a particular  
54 topic of concern include the effect on crops, food production, and trophic transfer (Gardea-Torresdey  
55 et al., 2014; Ma et al., 2018; White et al., 2022).

56 The interplay between plant growth, dissolution, evaporation, and aggregation are key aspects of the  
57 dynamic behavior of ENMs in the environment. Directional aggregation can result in the formation  
58 of larger particles with a more complex morphology. However, given the complexity of natural  
59 environments, most nanomaterials can be found in hetero-aggregated composites of different  
60 inorganic and organic materials (Judy et al., 2012; Ma et al 2018). These aggregates can be very  
61 different from original simple pristine morphologies and may even form highly branched structures  
62 similar to fractals, all of which subsequently dramatically affect their reactivity and transport (Ma et  
63 al., 2018; Huangfu et al., 2019).

64 Sectors with a large nanomaterial application such as medicine and food production may experience  
65 greater risks of ENMs exposure due to their uses, with thousands of tons of ENMs that are eventually  
66 discarded into the three main environmental matrices: soil, water, and air (Keller et al., 2013;  
67 Zuverza-Mena et al., 2017). ENMs with the greatest historical use include nanoscale ceria ( $n\text{CeO}_2$ ),  
68 silica ( $n\text{SiO}_2$ ), titania ( $n\text{TiO}_2$ ), as well as nanoscale copper oxide ( $n\text{CuO}$ ), zinc oxide ( $n\text{ZnO}$ ) and  
69 nanosilver ( $n\text{Ag}$ ), and as such, release in the environment has been investigated (Keller & Lazareva,  
70 2014; Mitrano et al., 2015). Considering the long-time span of use of select materials (1950 to 2050),  
71 efforts to estimate ENMs release through commercial and associated activity into the environment  
72 have been undertaken. For example, for  $n\text{SiO}_2$ , a global production between 100,000 and three million  
73 tons per year has been estimated, while for  $n\text{CeO}_2$ , levels likely reach the upper limit of 10,000 tons  
74 per year, and for  $n\text{Ag}$ , the literature reflects a production volume below 1,000 tons per year (Giese et  
75 al., 2018). The use of  $n\text{TiO}_2$  for the inhibition of microbial proliferation in food is one of the most  
76 important ways to prolong the shelf life of packaged products (Abutalib & Rajeh., 2020). However,  
77 a panel from EFSA concluded that E171 ( $\text{TiO}_2$ ) can no longer be considered as safe when used as a  
78 food additive (EFSA Journal 2021).

79 Gottschalk et al., (2009) calculated environmental concentrations using a probabilistic LCA (life  
80 cycle analysis) of ENM containing products. The authors modelled nTiO<sub>2</sub>, nZnO, nAg, carbon  
81 nanotubes (CNT), and fullerenes for the U.S., Europe, and Switzerland. The concentrations in the  
82 environment were calculated through probabilistic density functions and compared to ecotoxicology  
83 data. In the simulations, the values ranged from 0.003 ng L<sup>-1</sup> (fullerenes) to 21 ng L<sup>-1</sup> (nTiO<sub>2</sub>) for  
84 surface waters and from 4 ng L<sup>-1</sup> for fullerenes to 4 µg L<sup>-1</sup> for nTiO<sub>2</sub> for sewage treatment effluents.  
85 In Europe and the U.S., ENMs increased annually in sludge treated soil, and ranged from 1 ng kg<sup>-1</sup>  
86 for fullerenes to 89 µg kg<sup>-1</sup> for nTiO<sub>2</sub> (Gottschalk et al. 2009; Keller et al., 2013; Keller & Lazareva,  
87 2014; Rincon, 2019).

88 Importantly, quantum dots (QDs), as well as many carbon- and metal-based ENMs, have been shown  
89 to produce negative effects on animals and plants as a function of dose, including accumulation,  
90 alteration of physiological and biochemical parameters, and reduced growth or yield (Oh et al., 2016;  
91 Zuverza-Mena et al., 2017). Quantum dots have shown to enter plant roots and to damage the cell  
92 wall, dysregulating metabolism (Marmioli et al., 2020). While mercaptoacetic acid (MAA)-coated  
93 CdSe/ZnS QDs induced minimal toxicity on maize seedlings, pristine cadmium/tellurium (Cd/Te)  
94 QDs induced chromatin stress, mitochondrial damage and inhibition on green gram sprouts  
95 (*Phaseolus radiatus* L.) growth (Song et al., 2013). There are several routes by which ENM can enter  
96 the soil-plant system. These include agricultural application of sewage sludges which often contain  
97 nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, and nAg; as well as the application of nano-enabled agrochemicals, resulting in  
98 the direct entry of nSiO<sub>2</sub>, nTiO<sub>2</sub>, nZnO, nFeOx, nCuO, CeO<sub>2</sub> and nAg into agricultural soils (Lv et  
99 al., 2019; Verma et al., 2022).

100

### 101 *1.2. Conceiving and studying the ENM biotransformation*

102 Environmental and soil physico-chemical characteristics may significantly impact on ENMs  
103 aggregation, and dissolution, which may modify ENM bioavailability, uptake, translocation, and  
104 accumulation into terrestrial plants. In fact, light and temperature may induce potential changes on

105 the ENM structure, such as during foliar spray (Hong et al., 2021). Once within plant tissues, ENM  
106 biotransformation may alter particle stability and behaviour in terms of interactions with  
107 biomolecules, triggering differential plant defense mechanisms (Ma et al., 2018; Rawat et al., 2018).  
108 ENMs are subject to a range of processes that may lead to their partial dissolution or result in  
109 structural modifications (Milosevic et al., 2020; Marmioli et al., 2020). A schematic representation  
110 is reported in Figure 1. Nanoparticle biotransformation is a highly complex and poorly understood  
111 series of events and has been shown to occur during weathering in the soil, trophic transfer, and  
112 translocation within plant tissues. These reactions are highly dynamic and alter the original pristine  
113 structure of the nanoparticles in a number of ways, potentially causing the release of ions, but also  
114 the consequent restructuring (or destructuring) of the nanoparticle (Servin et al., 2017a).  
115 Biotransformation of nanomaterials may rest on the interaction with biological molecules that  
116 stabilize their external reactivity, such as peptides including those involved in detoxification, (*e.g.*,  
117 glutathione), fatty acids, secondary metabolites, and even components of cell membranes (Marmioli  
118 et al., 2020). Particle properties such as size, stability, charge, and dissolution may strongly influence  
119 other biotransformation mechanisms, potentially promoting enzymatic modification and  
120 functionalization with proteins (*e.g.*, corona protein) present in the cytoplasm and organelles (Ma et  
121 al., 2018; Marmioli et al., 2020). ENMs may maintain crystal structure when internalized by cells or  
122 may be disassembled and converted into less complex structures (by biological modification or  
123 chelation), thus reducing toxicity, and the risk of their accumulation and translocation (Wang et al.,  
124 2022). These post-uptake structural modifications involve specific parameters such as bond distance  
125 with other atoms or nature of the ligand atoms. In consideration of this, one objective in  
126 biotransformation studies is to investigate the physico-chemical forms (*e.g.*, nanocrystal structure)  
127 within exposed tissues and to characterize the structural differences within the new biotransformed  
128 molecules, including identification of the biomolecules interacting with the ENMs (Castillo-Michel  
129 et al., 2017; Marmioli et al., 2020). It has furthermore to consider how genetic diversity across  
130 different plant species and within the same plant species (in different cultivars) may influence the

131 ENM uptake and translocation (Deng et al., 2020). Some of the more interesting discoveries on the  
132 biotransformation and localization of metal based-ENMs into plants have been achieved with to the  
133 synchrotron-based techniques of imaging, elemental speciation, and atomic neighbors' identification.  
134 This transformation will be also influenced by the environment, these reactions of the same particle  
135 will be different on the leaf surface, in the mesophyll, in the vascular tissue, in different organelles,  
136 in the roots and in the rhizosphere (Sarret et al. 2013; Castillo-Michel et al., 2017).

137 Micro-X-Ray Fluorescence ( $\mu$ -XRF) and micro-X-Ray Adsorption Spectroscopy ( $\mu$ -XAS) K-, L- or  
138 L<sub>III</sub>-edge EXAFS and XANES spectra have be used to study the biotransformation of coated  
139 nanomaterials present in plants and soil (Lopez-Moreno et al., 2010; Judy et al., 2012).  $\mu$ -XRF is  
140 used for qualitative elemental analysis of heterogeneous biological samples. The interaction of the  
141 sample with high-energy X-ray radiation, which leads to X-ray absorption and emission of the  
142 fingerprint X-ray spectra for each element, is the key feature of this powerful analytical method. The  
143 absorption/excitation effect and relaxation process lead to atoms emitting fluorescence photons  
144 characterized by elemental specific energy (Chebakova et al., 2021). Extended X-ray absorption fine  
145 structure (EXAFS) is a technique that utilizes oscillations of the X-ray absorption coefficient on the  
146 high-energy side of an absorption edge. Such oscillations can reach up to 1000 eV above the edge  
147 and may have a magnitude of 10% or more. In addition, it is necessary to consider that atoms are not  
148 stationary. Thermal vibrations will obscure the EXAFS oscillations, and in the harmonic  
149 approximation, this can be accounted for by considering a Debye-Waller-type term. This led to a  
150 great improvement in the theoretical understanding of EXAFS and it is now established that a single  
151 scattering short-range order theory is adequate under most circumstances (Gurman, 1995). In  
152 addition, electrons that have undergone inelastic losses will not interference in the process. This is  
153 considered by an exponential damping term. It is the limited range of the photoelectrons in the energy  
154 region of interest 50-1000 eV that allows for a short-range order description of EXAFS also in  
155 crystalline materials (Gurman, 1995). The region which includes the pre-edge, edge-jump and post-  
156 edge to approximately 30-50 eV is referred to as the X-ray Absorption Near Edge Structure

157 (XANES), which describes the structural component of the X-ray absorption near-edge as an  
158 extension of the EXAFS, largely due to the long mean free path of the photoelectron and the  
159 dominance of high order multiple scattering contributions (Gräfe et al., 2014).

160 Synchrotron-based techniques use photons, which do not have mass; therefore the factor 1.22 is  
161 substituted by the non-relativistic mass of the electron which is  $m=5.485 \cdot 10^{-4}$  Da. This makes the  
162 resolution even smaller and increases penetration depth into the sample. On the other hand, EDX  
163 depends on the acceleration voltage of the particle or of the photon. Every element has its own orbital  
164 energies, and the acceleration voltage allows excitation one or more of these, independently if it  
165 comes from a TEM or from a synchrotron (Goldstein et al., 2003).

166 From the perspective of application,  $\mu$ -XRF can provide information on the presence and localization  
167 of specific elements within tissues, while XANES and EXAFS spectroscopy can provide information  
168 related to the valence state and coordination environment of the element of interest, as well as the  
169 molecular species present in the sample. The use of  $\mu$ -XRF and  $\mu$ -XANES for the analysis of  
170 nanoparticles in plants have been thoroughly reviewed by Castillo-Michel et al. (2017).

171 Importantly, these powerful methodologies open the possibility to mechanistically address many  
172 important environmental issues, such as the chemical activities of environmental pollutants, to trace  
173 environmental elemental cycles, element speciation in complex matrices, and to characterize the  
174 natural/anthropogenic complex matrixes that are not amendable for standard analytical and structural  
175 analyses (Puri et al., 2019). In many synchrotrons around the world, there is increasing use of  
176 dedicated beamlines to study environmental and plant tissues exposed to contaminants such as ENMs  
177 (Proux et al., 2017; d'Acapito et al., 2019).

178

179 The present review aims to describe the current understanding of metal based-ENMs  
180 biotransformation mechanisms in plants and focuses on correlating available physiological and  
181 molecular data with the information obtained by synchrotron-based techniques. This evaluation not  
182 only highlights biotransformation as one of the major driving forces mediating the biological effects

183 of ENMs on plants, but also offers some perspective on intentional and safer-by-design strategies that  
184 can ensure more sustainable application of these materials. Moreover, the study on plants is  
185 instrumental to the application of the REACH normative within European Union for toxicological  
186 and ecotoxicological studies (Replacement, Reduction and Refinement). Plants are higher eukaryotes,  
187 characterized by large nuclear genomes and organellar genomic information (within chloroplasts and  
188 mitochondria) that provide an effective model for many complex species (Chang et al., 2016).

189

190

## 191 ***2. Physiological and molecular effects as indirect evidence of ENM biotransformation in plants***

192 The physiological behaviour and related molecular pathways of response are important to  
193 characterizing and understanding ENM biotransformation. An important part of this involves  
194 comparing the effects of a nanomaterial with that of the bulk and ionic counterparts, as well as by  
195 investigating different exposure times and by exposing different plant organs and tissues (Schwab et  
196 al., 2016; Marmioli et al., 2021). Studies have added significant molecular data on the effects of  
197 ENMs exposure in plants (Schwab et al., 2016; Ma et al., 2018). Different results are often observed  
198 for the same element as a function of its form or size, *i.e.* nanostructured, bulk, or ionic species  
199 (Pagano et al., 2016; Wang et al., 2022). Detailed study of differential transcriptional regulation,  
200 protein abundance or metabolomic profiling (Huang et al., 2018; Majumdar et al., 2019; Gallo et al.,  
201 2021) are critical to demonstrating the nano-specificity of plant response. The study of organellar  
202 genome stability and the related stoichiometric variations during ENM treatment has also provided  
203 important mechanistic insight into plant to ENM exposure (Pagano et al., 2022). Advanced  
204 synchrotron-based techniques may either help to systematically understand the nano-bio interactions,  
205 with regard to physical and chemical reactions at the biomolecular surface: biomolecules may interact  
206 with ENMs, generating biomolecular corona, which change the ENM surface properties, and interfere  
207 with its functionality/reactivity (Hameed et al., 2022).

208 Regarding the physiological effects of ENMs on plants of agronomic interest, these studies have  
209 provided a better understanding of the specific properties of the ENMs that may enable sustainable  
210 use in the agrifood sector. Beyond the potential adverse effects upon bioaccumulation from soil or  
211 other exposure routes, there is an increasing interest in exploiting the potential positive effects of  
212 ENMs on plants, aiming to improve crop yields and quality. A range of mechanisms, including direct  
213 use as nanofertilizers (Verma et al., 2022), nanocarriers (Karny et al., 2018), smart delivery systems  
214 (Xu et al., 2022) or when in association to plant growth-promoting bacteria, are considered (Prado de  
215 Moraes et al., 2021). In addition, ENMs may act indirectly by protecting plants from biotic (*e.g.*,  
216 nanopesticides) or abiotic stressors (*e.g.*, wastewater and soil treatment) (Liu et al., 2015; Kah et al.,  
217 2018; Kumari et al., 2019). Due to the many variables involved, it is essential to obtain robust safety  
218 data regardless of the end use: ENM type, the modes and time of exposure, concentrations tested, and  
219 the plants used are all important considerations. Any recurring effects that occur under these different  
220 conditions are of particular interest and are explored below (see Figure 2 and Table 1). Table 2  
221 summarizes the major outcomes related to metal based-ENM biotransformation in plants, including  
222 the principal mechanisms involved, and the major physiological and molecular insights observed  
223 from exposure.

224

### 225 2.1. Lanthanides based ENMs

226 Cerium Oxide ( $n\text{CeO}_2$ ) has shown significant potential for agricultural applications, largely due to its  
227 properties as an ROS scavenger (Ma et al., 2015; Servin et al., 2017b). While  $\text{CeO}_2$  as a bulk crystal  
228 mainly consists of Ce(IV), the reduction to  $n\text{CeO}_2$  significantly enhances the relative amount of  
229 Ce(III), resulting in a higher catalytic effects comparable to the capabilities of a biological antioxidant  
230 (Eriksson et al., 2018). Servin et al. (2017b) used  $\mu\text{-XRF}$  and  $\mu\text{-XANES}$  to analyze the interactions  
231 between  $n\text{CeO}_2$  and different biochars in soil, observing that much of the Ce remained in  $n\text{CeO}_2$  form  
232 within the plant tissues. The dissolution rate of the nanoform can increase in acidic environments to  
233 generate Ce(III), as reported by Hernandez-Viezcas et al. (2013) who analyzed in *Glycine max* L. the



234 effects of nCeO<sub>2</sub> (1000 mg L<sup>-1</sup>, 48d exposure). Results have been confirmed by Rui et al. (2015), who  
235 used XANES on exposed cucumber (*Cucumis sativus* L.) tissues (2000 mg L<sup>-1</sup>, 21d exposure) to  
236 observe nCeO<sub>2</sub> association with phosphate. These properties highly impact not only reactivity but  
237 also nCeO<sub>2</sub> translocation. In zucchini (*Cucurbita pepo* L.), treated with 500 mg L<sup>-1</sup> of nCeO<sub>2</sub>, the  
238 nanoform is mainly present in the roots and stems, with limited translocation to the leaves (Pagano et  
239 al., 2016). However, co-contamination with other ENMs (e.g., CdS QDs) under same experimental  
240 conditions resulted in increased translocation to the shoots from 1000 to 3000 mg kg<sup>-1</sup> (Pagano et al.,  
241 2017). Similar results have been reported in several plant species: for example, Rossi et al (2017)  
242 nCeO<sub>2</sub> under co-exposure with ionic Cd in soybean (*Glycine max* L.) showed an altered (1-2 fold  
243 increased) translocation to the shoots. Interestingly, bulk CeO<sub>2</sub> translocation resulted similar to the  
244 nano-form, whereas ionic Ce was translocated in greater amounts to the shoots (Pagano et al., 2016).  
245 This analysis was supplemented with molecular data; the transcriptional profiles were evaluated in  
246 *C. pepo* and *S. lycopersicum* as a function of nCeO<sub>2</sub>, nLa<sub>2</sub>O<sub>3</sub> and nCuO exposure and were compared  
247 with bulk and ionic forms using a set of 38 genes based on the *A. thaliana* orthologs as potential  
248 biomarkers of exposure/effects (Marmioli et al., 2014). The responses observed were generally  
249 different in term of up- or down-regulation as a function of Ce form (Pagano et al., 2016). Of  
250 particular interest are impacts on the chloroplast are the *PetL* and *PSBN* genes, which encode for a  
251 structural component of the cytochrome b<sub>6</sub>f complex and low molecular weight protein located on  
252 thylakoid membrane as a component of the photosystem II (PSII), respectively (Figure 2, Table 1).  
253 These two chloroplastic genes were differentially expressed across nano-, bulk, and ionic forms. A  
254 similar trend was also evident for nLa<sub>2</sub>O<sub>3</sub> and nCuO exposure scenarios. Interestingly, by analyzing  
255 the effects on chloroplast and mitochondrial genomes in *A. thaliana* in terms of copy number, the  
256 effects of nCeO<sub>2</sub> and CeCl<sub>3</sub> exposure were rather limited as compared to the untreated control, which  
257 agrees with the limited translocation to the shoots (Pagano et al., 2022). With regard to proteomic  
258 analysis, Majumdar et al. (2015) conducted a quantitative proteomic analysis of kidney beans  
259 (*Phaseous vulgaris* L.) seeds after nCeO<sub>2</sub> exposure and reported that the major seed proteins

260 associated with nutrient storage (phaseolin) and carbohydrate metabolism (lectins) were significantly  
261 reduced by nCeO<sub>2</sub> (62.5-500 mg kg<sup>-1</sup>, 50d exposure) in a dose dependent manner. Interesting, the  
262 plants did not exhibit overt toxicity.

263 In fact, at the physiological level cerium-based nanoparticles generally do not produce phytotoxicity  
264 (Ma et al., 2015; Rui et al., 2014; Lizzi et al., 2020; Rodrigues et al., 2021), though some have  
265 highlighted a positive impact on biomass and on physiological indicators such as chlorophyll and  
266 photosynthesis at selected doses (Rossi et al., 2017; Gui et al., 2017). Another important aspect of  
267 nCeO<sub>2</sub> seems to involve enhance tolerance to saline stress, leading to improved phenotypic and  
268 enzymatic performances and ROS elimination in seeds priming (An et al., 2020; Liu et al., 2021;  
269 Hassanpouraghdam et al., 2022; Chen et al., 2022).

270 Compared to nCeO<sub>2</sub>, nanoscale lanthanum oxide (nLa<sub>2</sub>O<sub>3</sub>) exhibits lower stability, increased ion  
271 dissolution, greater translocation from roots and shoots, all of which seems to lead to higher  
272 phytotoxicity. The limited stability of nLa<sub>2</sub>O<sub>3</sub>, as compared to nCeO<sub>2</sub>, has been confirmed by  $\mu$ -XRF  
273 analysis in *Cucumis sativus* L. through element speciation, dissolution studies in aqueous solution  
274 and *in planta*. After 14d treatment, the nCeO<sub>2</sub> structure in the roots remains mostly preserved (more  
275 than 80%) while pristine nLa<sub>2</sub>O<sub>3</sub> structure was observed at levels below 10% (Ma et al., 2015).

276 Interestingly, co-contamination with nCeO<sub>2</sub> strongly reduces the uptake of nLa<sub>2</sub>O<sub>3</sub> (Pagano et al.,  
277 2017). The different behaviour of the two ENMs was evident in the transcriptomic profile: only 7 out  
278 of 38 genes were commonly modulated between nCeO<sub>2</sub> and nLa<sub>2</sub>O<sub>3</sub>; these genes were involved in  
279 primary metabolic functions, protein synthesis and stress response (Pagano et al., 2016). Several  
280 publications using different model plants were compared, and the reported effects due to nLa<sub>2</sub>O<sub>3</sub>  
281 exposure in soil include reduction in root and leaf biomass (Ma et al., 2015), decreased transpiration  
282 (Yue et al., 2019), decreased photosynthesis (Xiao et al., 2021) and reduced pigment concentration  
283 (Neves et al., 2019). The decrease in photosynthetic activity is also reflected by altered root  
284 morphology, including root cracking (Xiao et al., 2021) and the presence of apoplastic barriers (Yue  
285 et al., 2019). Interestingly, the adverse effect of nLa<sub>2</sub>O<sub>3</sub> on plant biomass was alleviated under binary

286 exposure combinations with ENMs such as nCuO and nZnO (Pagano et al., 2017). As ideal case  
287 study, information on RedOx state and potential translocation of lanthanide-based nanoforms within  
288 plant tissues become fundamental in the mechanistic understanding on the physiological and  
289 molecular effects, with regard to application in the agrifood sector.

290

## 291 2.2. Titanium oxide ENM

292 Titanium dioxide (nTiO<sub>2</sub>) has been largely studied as a potential environmental and agricultural  
293 contaminant (Servin et al., 2012; 2013). nTiO<sub>2</sub> has demonstrated a high stability, both in anatase and  
294 rutile crystal form (Servin et al., 2012). Translocation of nTiO<sub>2</sub> (0-750 mg kg<sup>-1</sup>, 150d exposure) from  
295 soil to roots and to shoots in *C. sativus* is generally limited, though the two crystal structures were  
296 evident in the leaf trichomes and fruit by  $\mu$ -XANES spectra (Servin et al., 2013). Ruotolo et al. (2018)  
297 analyzed and reviewed the molecular responses of *A. thaliana* and other model species to nTiO<sub>2</sub> and  
298 reported that exposure triggers an abiotic stress response at the transcriptomic level, involving ROS  
299 detoxification systems, triterpenoid and phenylpropanoid metabolism, and hormone signaling  
300 pathways involving in the response to salicylic acid, jasmonic acid, ethylene, and brassinosteroids.  
301 At the post-transcriptional level, several miRNAs were strongly modulated, including miR395 and  
302 miR399 as key regulators of plant adaptive responses to nutrient starvation (Pagano et al., 2021).  
303 Thus, the ability of nTiO<sub>2</sub> to modulate ROS signaling is particularly effective under abiotic stress  
304 conditions. Here, the presence of this ENMs enhances plant physiological parameters by stimulating  
305 the activation of several defense mechanisms. Several studies (in plants such as *C. sativus*, *S.*  
306 *lycopersicum*, *V. faba*) have shown that in both saline soils and under drought conditions, the addition  
307 of nTiO<sub>2</sub> increases root length, plant biomass, and other parameters such as H<sub>2</sub>O<sub>2</sub> level, antioxidant  
308 activity, sugar content, and chlorophyll amount (Servin et al., 2012; Nasir Kahn, 2016; Abdel Latef  
309 et al., 2018; Mustafa et al., 2021). However, higher concentrations can result in phytotoxicity, likely  
310 due to aggregation and subsequent excessive ROS production (Mattiello et al., 2015; Gohari et al.,  
311 2020). However ROS are “double blade” sword because they can also trigger production of defensive

312 molecules as shown recently by Castro et al., (2021). Interestingly, when either considering the  
313 utilization of pristine and coated nTiO<sub>2</sub> (hydrophilic or hydrophobic) in carrot (*Daucus carota* L.),  
314 responses observed depended mainly on the nTiO<sub>2</sub> surface coating, concentration and in soil  
315 weathering (Wang et al., 2021a; 2021b). Taproot and leaf fresh biomass and plant height were all  
316 increased with exposure, as well as nutrient uptake (Fe in leaves; Mg in taproots; Ca, Zn, K in roots).  
317 Conversely, sugar and starch contents were negatively affected, compromising the nutritional quality  
318 (Wang et al., 2021b).

319

### 320 2.3. Gold and silver nanoparticles

321 Similar to nTiO<sub>2</sub>, gold nanoparticles (nAu) are highly stable in plants: nAu remained mostly as Au<sup>0</sup>  
322 within the plant tissues (*Nicotiana tabacum* L. cv. Xanthi nc.), even if accumulated and translocated  
323 (Sabo-Attwood et al., 2011). Specifically, XANES analyses demonstrated that nAu maintained its  
324 nanoparticle structure without any biotransformation or ionic release. There are no actual uses for  
325 gold nanoparticles and plants, it just used as a tool to study NP-plant interactions. nAu levels in  
326 biosolids would ever be high enough to be considered phytotoxic. It is known that nAu toxicity  
327 depends on concentration, particle size and shape: nAu with a smaller particle size (3.5nm,  
328 concentration of 48 mg L<sup>-1</sup>) were evenly biodistributed across the plant in comparison with the  
329 18.5nm nAu (in a concentration of 76 mg L<sup>-1</sup>), even leading to the formation of necrotic leaf lesions  
330 and plant death after 30 days (Sabo-Attwood et al., 2011). Other studies have shown that nAu  
331 exposure improved radical scavenging and antioxidant enzymatic activities and modulated miRNA  
332 expression implicated plant abiotic stress response (miR398, miR408). In particular, the regulation  
333 of superoxide dismutase (SOD) led to an increased ROS scavenging activity, root elongation,  
334 seedling growth, and seed yield (Arora et al. 2012; Kumar et al., 2013; Siddiqi & Husen, 2017).  
335 Given the widespread commercial utilization and environmental relevance (e.g., wastewater  
336 treatment; fertilization) of silver nanoformulations (nAg), the effect on plant species has been a topic  
337 of robust study. Stegemeier et al. (2015) analyzed the nAg and nAg<sub>2</sub>S speciation in *Medicago sativa*

338 L., demonstrating that nAg accumulates in the root elongation area but that nAg<sub>2</sub>S remains adhered  
339 to the root surface; Ag ions accumulate more uniformly throughout the root tissues. Notably, the Ag  
340 accumulation in the root apoplast was determined by XRF. The presence of nAg in root cell walls  
341 demonstrated the uptake of partially dissolved nAg and translocation along the apoplast. Larue et al.  
342 (2014) localized and determined nAg speciation in *L. sativa* after foliar spray treatment through  $\mu$ -  
343 XRF and  $\mu$ -XAS techniques; the authors reported that nAg was able to cross the foliar cuticle,  
344 penetrating in the leaf tissue through the stomata. Moreover, nAg biotransformed through oxidation  
345 and complexation with thiol-containing molecules such as glutathione (GSH). These findings  
346 correlated well with the transcriptomics analyses of *A. thaliana* exposed to different types of nAg:  
347 plant response included defensin-like proteins, plant thionin,  $\beta$ -glucosidases, cytochrome P450  
348 proteins, and glutathione-S-transferase (GST) members (Kaveh et al., 2013). Although some studies  
349 point out that the morphological and physiological effects of nAg exposure were strictly dependent  
350 on particle size and concentration and that sublethal concentrations may have also beneficial effects  
351 (Wang et al., 2013; Syu et al., 2014), most of reports demonstrated reduced root elongation and shoot  
352 biomass, together with decreased levels of chlorophyll, pigments, micronutrients, and increased level  
353 of ROS and activity of enzymes involved in the oxidative stress response (Yin et al., 2011; Zuverza-  
354 Mena et al., 2016; Yang et al., 2018; Lahuta et al., 2022).

355

#### 356 2.4. Iron-based ENMs

357 Iron-based nanomaterials, including iron oxides (nFeOx) and zero valent iron (nZVI), have been  
358 investigated in plant systems and the reports highlight two major routes of entry: i) a reductive and  
359 proton-promoted process able to modify the structure of the ENM or ii) through the secretion of plant  
360 transporters (*e.g.*, phytosiderophores) with a high affinity for Fe (III) (Morrissey & Guerinot, 2009).  
361 Dwivedi et al. (2018) investigated nZVI exposure in *C. sativus* and reported that transformed nZVI  
362 was stored in the root cell membrane and vacuoles of the leaf parenchyma. XAS identified ferric  
363 citrate and iron (oxyhydr)oxides as the main transformation products in roots and shoots, albeit in

364 different proportions. The major pathways of nZVI biotransformation involve interaction with low  
365 molecular weight organic acid ligands and on the dissolution/precipitation of the mineral products.  
366 Transcriptional analyses performed on H<sup>+</sup>-ATPase genes (*CsHA1*, *CsHA2*) showed an upregulation  
367 of these genes upon nZVI exposure (and relative root acidification), indicating that the plant-  
368 promoted transformation of nZVI can be driven by protons released by the roots.

369 A separate study investigated the effects of nFe<sub>2</sub>O<sub>3</sub> and nFe<sub>3</sub>O<sub>4</sub> on *A. thaliana*, highlighting  
370 differences in the response between nanoparticle forms and metal salts through a nanoscale-specific  
371 response pathway involving energy production and oxidative stress. The differential response was  
372 ascribed to the ENM and the metal salt dissolution rates and the toxicity of the metal ion, which is  
373 more compatible with biotransformation processes in the plant tissues. Importantly, specific effects  
374 on plastid and mitochondrial genomes were evident, with nFeOx causing a 1- to 3-fold increase in  
375 ptDNA and mtDNA copy numbers depending on the stability of the nanoform utilized (Pagano et al.,  
376 2022).

377 Given their widespread application in soil and water remediation, a primary concern with iron  
378 nanoparticles is a potential toxicity from excessive accumulation in the environment. However,  
379 several studies have shown that plant exposure to this type of nanoparticle does not result in  
380 phytotoxicity. For example, Dwivedi et al. (2018) evaluated the potential environmental impact of  
381 nZVI on *C. sativus* in soil and in hydroponic culture, and reported no instances of reduced plant  
382 biomass even at the highest doses (from 250 to 1000 mg L<sup>-1</sup>) and for *O. sativa*, the low doses (50-500  
383 mg L<sup>-1</sup>) of nZVI and nFe<sub>3</sub>O<sub>4</sub> improved plant growth (Li et al., 2021). The use of this nanomaterial as  
384 a soil conditioner for remediation of metal-contaminated soils is confirmed by the demonstration of  
385 improved plant growth in Cd-contaminated soils (Rizwan et al., 2019; Manzoor et al., 2021);  
386 mechanistically, this involves limiting cadmium translocation and the promotion of antioxidant  
387 activity.

388 In summary, the extent and the degree of biotransformation of nZVI, which consists in the  
389 biochemical alteration of chemical compounds within a living tissue, are reflected in the physico-  
390 chemical properties, macromolecular interaction, and biologically mediated pathways observed.

391

### 392 2.5. Zinc-based ENMs

393 Zinc-based nanomaterials have been applied to plants to increase food safety, promote food  
394 production and enhance sustainability by reducing oxidative stress symptoms induced by abiotic  
395 stressors (Faizan et al., 2021). nZnO is characterized by a low stability, and a high dissolution rate  
396 (Lv et al., 2021). Hernandez-Viezcas et al. (2013) exposed *Glycine max* L. to nZnO (500 mg kg<sup>-1</sup>,  
397 48d exposure):  $\mu$ -XRF analysis showed no detectable ZnO NPs within the tissues, while  $\mu$ -XANES  
398 data showed O-bound Zn in a form resembling Zn citrate. Lv et al. (2015) studied the effects of nZnO  
399 in *Z. mays* L. and used  $\mu$ -XANES to demonstrate that the majority of accumulated Zn was derived  
400 from Zn<sup>2+</sup> released from the nanoparticles and was accumulated mainly as Zn phosphate in epidermis,  
401 cortex, and root tip cells. The results were correlated to transcriptomic analyses in which gene  
402 ontology (GO) performed in nZnO-exposed *A. thaliana* revealed significant commonalities with the  
403 response to Zn<sup>2+</sup> ions, particularly with proteins involved in metal binding, transport, metal  
404 homeostasis and detoxification. This suggests that Zn ion release by nZnO is a key in mediating the  
405 overall effect on plant species (Landa et al., 2015). These findings have been extended to other  
406 species, such as *C. pepo* L.; here nZnO treatment was shown to modulate genes that encode for  
407 transporters of heavy metals, cellular response to abiotic stress, decreased chlorophyll production,  
408 and induction of secondary metabolite biosynthesis (Pagano et al., 2017).

409 In recent years other forms of Zn-based nanomaterials have been tested for a potential plant  
410 remediation purpose, such as ZnS QDs (Imperiale et al., 2022). An analysis of the effect of ZnS QDs  
411 and ionic Zn exposure on mitochondrial and plastid genome copy number demonstrates that both  
412 increase by 1 to 3-fold), but that ZnS QDs dissolution alone does not explain the phenomenon; this  
413 suggests that ZnS QDs biotransformation may occur within the plant tissues and organs to a form

414 more similar to ionic than nanoscale Zn (Pagano et al., 2022). Zinc-based nanomaterials have also  
415 shown interesting properties as nanofertilizers, including mitigating abiotic and biotic stress (*e.g.*, salt  
416 stress, infections), regulating micronutrient uptake, improving water use efficiency, and promoting  
417 detoxification of heavy metals (Akhtar et al., 2021; Zafar et al., 2022). Under drought conditions, the  
418 nZnO (5 mg kg<sup>-1</sup>) significantly increased grain yield in sorghum (*Sorghum vulgare* Moench) and fruit  
419 yield in eggplant (*Solanum melongena* L.), respectively by 22-183% and 12-23% (Dimkpa et al.,  
420 2019; Semida et al., 2021).

421

## 422 2.6. Copper oxide

423 Copper oxide nanomaterials (nCuO) are among the most utilized ENMs with plants, including use as  
424 a nanopesticide or nanofertilizer (Elmer et al., 2018; Lowry et al., 2019; Xu et al., 2022). nCuO  
425 dissolution within the plant tissues has been demonstrated (in *C. pepo*), and this was shown to depend  
426 not only on uptake, and translocation, but also on the interaction with important biomolecules (Tamez  
427 et al., 2019; Marmiroli et al., 2021).

428 EXAFS (Marmiroli et al., 2021) demonstrated that the local Cu environment in the higher shells  
429 shows small differences between roots and flowers. A second Cu-O shell path was present in both  
430 flowers and roots; a Cu-Cu bond was also observed in roots but was not observed in flowers. A full  
431 transcriptomics analysis by RNAseq was performed to highlight the differential responses between  
432 nano-, bulk and ionic forms in roots, leaves and pollen (Marmiroli et al., 2021). The results  
433 highlighted the nano-specificity of the responses; the modulated genes (significantly up- or down-  
434 regulated genes) observed were more significant in the roots and decreased with translocation to  
435 leaves and pollen. However, the portion of the response common to the three Cu forms tested was  
436 shown to increase following the translocation from roots to shoots (Marmiroli et al., 2021). A  
437 characterization of the main steps and implications involved in this phenomenon, as well as some  
438 relevant biomarkers observed in different plant species, is presented in Figure 2 (details reported in  
439 Table 1).



440 Additional data was presented by Servin et al. (2017a), who studied nCuO weathering in *Lactuca*  
441 *sativa* L.: lettuce was exposed to unweathered and 70d-weathered nCuO, and corresponding bulk and  
442 ionic form (0–400 mg kg<sup>-1</sup>) for 70 d in soil. To assess nCuO trophic transfer, leaves were fed to  
443 crickets (*Acheta domestica* L.) as primary consumer, followed by insect feeding to lizards (*Anolis*  
444 *carolinensis* L.) as secondary consumer, in both cases for 15d. The authors used  $\mu$ -XANES to show  
445 that Cu(II) was reduced to Cu(I) within the plant roots, and used a transcriptional analysis of to show  
446 that several biomarkers, including *CCH* and *COPT5*, which encodes for a copper chaperon and a  
447 copper ion transporter, respectively, were significantly decreased by weathering.

448 In spite of being widely used, results regarding the physiological effects upon nCuO exposure are  
449 rather discordant. For example, Deng et al. (2022a) reported that, unlike the bulk counterpart, nCuO  
450 (0-600 mg kg<sup>-1</sup> of soil) does not produce toxicity in rice (*O. sativa*), but rather improves the supply  
451 of essential elements, including increasing content of sugar and starch, as well as overall yield.

452 The grain of weedy and cultivated rice were differentially impacted by nCuO, bulk or ionic forms,  
453 showing also a cultivar-specific and concentration-dependent response. Cu translocation directly  
454 influenced plant yield, sugar production, starch content, protein content, and expression of auxin  
455 associated genes in grain (Deng et al., 2022b). Analyzing the effect of citric acid (CA) coated copper  
456 oxide (CA-nCuO) and its application (foliar spray or soil exposure) on the growth and physiology of  
457 soybean (*Glycine max* L.), nCuO appeared to be more accessible for plant uptake, as compared to  
458 CA-nCuO, decreasing the protein content, and inhibiting plant growth. CA reduced CuO NPs  
459 toxicity, demonstrating that surface modification may change the toxic properties of NPs (Deng et  
460 al., 2022c).

461 Treatment of *Lactuca sativa* L. with nCuO significantly increased biomass as compared to CuO  
462 microparticles (Wang et al., 2019). In addition, plants can benefit from nCuO treatment through  
463 enhanced defensive pathways, and through direct antimicrobial and antifungal activities (Elmer et al.,  
464 2018). For example, exposure of nCuO to *Solanum lycopersicum* increased root and stem length, leaf  
465 number, and chlorophyll content, and also inhibited the mycelial growth of *Fusarium oxysporum* sp.

466 Lycopersici (Lopez-Lima et al., 2021). Conversely, some authors report toxic and inhibitory effects  
467 on the growth in plants such as lettuce (*Lactuca sativa* L., 0-1000 mg L<sup>-1</sup>, 5-15d exposure by foliar  
468 spray), turnip (*Brassica rapa* L., 50-500 mg L<sup>-1</sup>, 14d exposure), and wheat (*Triticum aestivum* L., 50  
469 mg kg<sup>-1</sup> in sand, 1-14d exposure) upon nCuO treatment. The toxic effects are largely ascribed to the  
470 redox reactivity and ROS generation of the nanoparticle form (Dimkpa et al., 2012; Chung et al.,  
471 2019; Xiong et al., 2020). Others have reported no significant impact at the physiological level  
472 (Servin et al., 2017a; Tamez et al., 2019; Marmioli et al., 2021; Roubeau Dumont et al., 2022), which  
473 highlights the importance of the experimental variables and design, including dose, particle  
474 properties, exposure conditions and endpoints.

475

## 476 2.7. Quantum dots

477 Cadmium-based nanomaterials, and cadmium sulfide quantum dots (CdS QDs) in particular, have  
478 been used as a model material to elucidate physiological mechanisms and molecular pathways  
479 involved in the response plant response to exposure (Marmioli et al., 2014; Imperiale et al., 2022).  
480 A Systems biology approach gave a complete picture of the targets in both model (*A. thaliana*) and  
481 crop (*C. pepo*) species (Marmioli et al., 2014; Marmioli et al., 2015; Pagano et al., 2017; Gallo et  
482 al., 2021; Marmioli et al., 2020; Pagano et al., 2022). In *A. thaliana*, CdS QDs tolerant mutants were  
483 used to establish *in vitro* inhibition concentrations for growth (80 mg L<sup>-1</sup>) in an attempt to elucidate  
484 the mechanisms involved in the plant response; the results largely implicated metabolic functions and  
485 chloroplast energy production as sensitive targets (Marmioli et al., 2014). The results demonstrate  
486 that CdS QDs and ionic Cd were exploiting different pathways in the plant, highlighting that the  
487 tolerance to CdS QDs did not overlap with the tolerance to CdSO<sub>4</sub>. Conversely, Cd sensitive mutants  
488 of *Arabidopsis* (Howden & Cobbett, 1992) that were exposed to CdS QDs did not exhibit differences  
489 in growth as compared to the wild type line (Marmioli et al., 2014). A transcriptomic analysis and  
490 proteomic comparison between wild type and tolerant mutants highlighted that only a few genes were  
491 commonly modulated upon ionic Cd and CdS QDs treatment (Marmioli et al., 2015; Gallo et al.,

492 2021). Marmiroli et al., (2020) used EXAFS to investigate the cadmium environment in planta and  
493 showed that the spectra were compatible with a mixed O/S coordination; while Cd–S distances did  
494 not show relevant variations, Cd–O distances varied in samples grown with QDs compared with those  
495 grown with CdSO<sub>4</sub>. The number of Cd–S bonds in plants grown with QDs was higher than Cd–O  
496 bonds. This EXAFS analysis demonstrated that CdS QDs were biotransformed after uptake: the QD  
497 original structure was modified but not completely absent within the plant cell, and Cd atoms were  
498 not released as Cd ions. Interestingly, CdS QDs showed a relatively high stability; once accumulated  
499 by the plant, the QD may go through different stages in the response pathways: i) exposure: explained  
500 by the different genetic mechanisms behind the physiological/molecular response between the wild  
501 type and tolerant phenotypes; ii) reactivity/biotransformation: explained by a transition phase in  
502 which the structure of CdS QDs is modified to decrease particle reactivity, and this can be detected  
503 by XANES and EXAFS analyses; iii) effects/detoxification: transcriptomic, proteomic and  
504 metabolomic response related to the physico-chemical forms after QDs biotransformation.  
505 Additionally, the effects on organelle genomes (ptDNA and mtDNA) demonstrate how QDs  
506 biotransformation may modify the genomes stoichiometry or sub-stoichiometry, likely through  
507 potential morpho-functional adaptive response triggered by modifications in the bioenergetic redox  
508 balance, or a reduction of photosynthesis or cellular respiration rates after QD exposure (Pagano et  
509 al., 2022).

510 Similarly to what was observed in *A. thaliana*, CdS QDs induced analogous effects other plant species  
511 of agricultural interest: Pagano et al. (2017) analyzed the effects of the CdS QDs in a context of  
512 ENMs binary co-contamination, highlighting a similar response as in *A. thaliana*; specific and  
513 common biomarkers were involved between CdS QDs and other the ENMs tested (nCeO<sub>2</sub>, nLa<sub>2</sub>O<sub>3</sub>,  
514 nCuO, nZnO). Majumdar et al. (2019) investigated the effect of differently functionalized CdS QDs  
515 in *G. max*; the authors used proteomic and metabolomic endpoints to demonstrate how the  
516 transmembrane proteins involved uptake and related genes including *NRAMP6* and *HMA8* were  
517 differently regulated in CdS QDs and ion treated plants. In addition, ATP-dependent ion transporters

518 in the membranes presented feedback mechanisms in the soybean roots to restrict the uptake of CdS  
519 QDs and simultaneously to alter the mineral acquisition. Moreover, CdS QDs altered major metabolic  
520 functions, including glutathione metabolism, the tricarboxylic acid cycle, glycolysis, fatty acid  
521 oxidation and phenylpropanoid and amino acids biosynthesis. Physiologically, CdS QDs, induced  
522 oxidative stress, decreased biomass, reduced chlorophyll and carotenoids content, and damaged  
523 primary roots (Majumdar et al., 2019; Pagano et al., 2022).

524

525

### 526 ***3. Biotransformation as a perspective to comprehend ENM response in plant***

527 ENMs have been rather extensively tested in recent years, with data indicating that several physico-  
528 chemical parameters are fundamental to explaining their behaviour during exposure, including  
529 composition, stability, surface charge, and functionalization. These ENM properties become  
530 biologically relevant and mediate subsequent biotransformation processes, including: i) the  
531 possibility to be translocated within organs, tissues, and cells; ii) the ability to interact with the  
532 biologically active environment within the plant (*e.g.*, phospholipids, nucleic acids, proteins,  
533 secondary metabolites, reactive oxygen species); iii) the dissolution rate and the consequent ion  
534 release. Importantly, to fully comprehend the plant response to ENMs exposure, one must consider  
535 the biologically modified ENMs forms that are indicative of the highly complex interactions between  
536 plants and ENMs interaction. Integration of the information from physiological and molecular  
537 analyses with physical evidence (*e.g.*, types and number of atoms surrounding the ENM, radial  
538 distance between atoms of the interactors and atoms constituting the ENM; ENM crystal structure)  
539 obtained through high energy X-ray spectroscopy platforms such as synchrotron-based techniques  
540 will enable a more realistic, mechanistic, and systems-level picture of plant response to ENM  
541 exposure.

542 This review describes some of the primary biological constraints that determine ENM  
543 biotransformation in plants (Figure 2, Table 2). For ENMs characterized by high stability, such as

544 nCeO<sub>2</sub>, nTiO<sub>2</sub> or nAu, limited dissolution and translocation has been observed, even considering  
545 differences determined by structure and atomic properties related to the redox state (*e.g.*, the redox  
546 state of Ce). Conversely, ENMs with higher dissolution such as Fe- or Zn-based ENMs, nCuO, nAg  
547 or nLa<sub>2</sub>O<sub>3</sub>, exhibit greater translocation rates, likely involving a dynamic process of particle  
548 interaction with the plant biomolecules that increase ENM solubility and bioavailability, as  
549 exemplified with nCuO (Marmioli et al., 2021).

550 The importance of in planta ENM biotransformation is corroborated indirectly at molecular level by  
551 “omic” analyses that can describe the effects on the plant at genetic and epigenetic level (including  
552 genome stability) by measuring transcriptional modulation, protein abundance and metabolite  
553 synthesis, as well as on physiological (phenotypical) level by observing the plant redox state, ROS  
554 production, photosynthetic activity, and cellular respiration rate in response to stress (Marmioli et  
555 al., 2020; Gallo et al., 2021). The direct measurement of changes upon ENM biotransformation within  
556 the plant tissues by synchrotron-based techniques ( $\mu$ -XRF,  $\mu$ -XANES, and XAS) provide critical  
557 information in terms of distribution, atomic redox state, and atomic local structure, and add critical  
558 knowledge necessary to understand the ENM-plant interactions. This information is highly relevant  
559 with regard to potential applicability: ENMs can interact with sensitive ecosystem components within  
560 trophic food chains, affect microbial populations in soil, enter into the plant and where they can be  
561 translocated to different tissues and organs, including the edible tissues or organs (Holden et al., 2013;  
562 Liu et al., 2015). Biotransformation can occur at each step within these processes, modifying and/or  
563 amplifying ENM effects at organism level. These interactions from the level of ecosystem, organism,  
564 tissue, cell, and organelles become key factors when applying “ENM biotransformation” as a concept  
565 for a safer design, when considering applications for agriculture and food production, and for  
566 minimizing the adverse biological impact (Burello & Worth, 2015; Pagano et al., 2018; Lowry et al.,  
567 2019; Kah et al., 2019; Zulfiqar et al., 2019; Ma et al., 2020; Hameed et al., 2022; Xu et al., 2022).

568

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572

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578

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581

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#### 1094 **Figure captions and Tables**

1095

1096 Figure 1. Schematic representation of principal effects emerging after biotransformation of ENMs in  
1097 soil, on foliar surface and within the plant tissues. ENMs in soil, due to particle and soil physico-  
1098 chemical interactions, may remain unmodified, or may undergo to potential dissolution (with ions  
1099 release), or undergo homo-/hetero-aggregation, which may highly influence particle stability and  
1100 potential bioavailability (and consequently their uptake). On the foliar surface, temperature and light  
1101 may also affect particle stability before uptake into the leaf tissues. Once within the plant, ENMs  
1102 pristine, or modified, can interact with several biomolecule types (peptides, sugars, lipids, nucleic  
1103 acids, secondary metabolites) leading to phenomena such as coating, enzymatic degradation,

1104 chelation or functionalization, which may influence the biotransformed particle at level of  
1105 translocation, storage or reactivity. These parameters may also influence the interaction within the  
1106 plant cell, triggering differential responses (*e.g.*, toxicity, oxidative stress, ROS production), which  
1107 may be indirectly measured by physiological and molecular assays, but directly observed through  
1108 physical strategies, including synchrotron-based methods.

1109

1110 Figure 2. Principal effects of ENM biotransformation in plant and relevant biomarkers observed in  
1111 different plant species from model organisms (*A. thaliana*) to crops (*C. pepo*; *S. lycopersicum*; *G.*  
1112 *max*) and different tissues (roots, leaves and flowers/pollen). Relevant ENM parameters such as size,  
1113 stability, dissolution may influence the translocation from roots to shoots. Potential biotransformation  
1114 mechanisms that may occur within plant tissues are also reported: enzymatic degradation, protein  
1115 functionalization, functionalization at the level of cytoplasm and organelles (organic acids, thiol-  
1116 containing compounds, aminoacids, sugars, secondary metabolites). In this scenario, chloroplast  
1117 become not only a in important actor in the energy production but also one of the key targets and  
1118 main regulators involved in the ENM exposure and response. Details on the biomarkers generated  
1119 are reported in Table 1.

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1124 Table 1. Genes as potential biomarkers of exposure/effect observed in roots, leaves and pollen, in  
1125 different plant species (reported in Figure 2). Genes reported cannot be considered only as modulated  
1126 in the different plant organs by the different type of ENMs, but also they showed a nano-specificity  
1127 during the ENM response. It is also important to observe how, depending on the ENM type,  
1128 biotransformation, and as indirect consequence, the transcriptomic response can be convergent  
1129 between different forms (nano, bulk, ion) of the same element (see Figure 2).

flower & pollen					
plant	gene	function	pathway	ENM	reference
<i>Cucurbita pepo</i> L.	Cp4.1LG00g07430	Beta-galactosidase	primary metabolism	nCuO	Marmioli <i>et al.</i> , 2021
	Cp4.1LG13g06380	Pectinesterase	primary metabolism		
	Cp4.1LG12g04750	Phosphatidylinositol 3-/4-kinase family protein	primary metabolism		
	Cp4.1LG03g02560	Delta-1-pyrroline-5-carboxylate synthetase	primary metabolism		
	Cp4.1LG02g07240	Leucine-rich repeat family protein	signaling, stress response		
	Cp4.1LG02g12750	Protein EFR3 like	signaling		

leaf					
plant	gene	function	pathway	ENM	reference
<i>Cucurbita pepo</i> L.	Cp4.1LG01(*)	PSBN, photosystem II reaction center protein N	chloroplast electron transport	nCeO2 nLa2O3	Pagano <i>et al.</i> , 2016 Pagano <i>et al.</i> , 2017
	Cp4.1LG00(*)	PetL, component of Cytochrome b6f	chloroplast electron transport	nCuO nZnO CdS QD	
<i>Solanum lycopersicum</i> L.	Solyc09g074540	PetL, component of Cytochrome b6f	chloroplast electron transport	nCeO2 nLa2O3 nCuO	Pagano <i>et al.</i> , 2016
<i>Glicine max</i> L.	Glyma12g36130	PetL, component of Cytochrome b6f	chloroplast electron transport	CdS QD	Majumdar <i>et al.</i> , 2019
<i>Arabidopsi thaliana</i> L.	AtCg00590	PetL, component of Cytochrome b6f	chloroplast electron transport	CdS QD	Marmioli <i>et al.</i> , 2014
	AtCg00270	PSBN, photosystem II reaction center protein N	chloroplast electron transport		

root					
plant	gene	function	pathway	ENM	reference
<i>Cucurbita pepo</i> L.	Cp4.1LG16g08630	BIP3, Heat shock protein 70 family protein	protein folding, stress response	nCeO2 nLa2O3 nCuO nZnO CdS QD	Pagano <i>et al.</i> , 2016 Pagano <i>et al.</i> , 2017
	Cp4.1LG00g00090	GPT2, glucose-6-phosphate/phosphate translocator	primary metabolism		
	Cp4.1LG05g08050	RPS12, ribosomal protein S12A	protein synthesis		
	Cp4.1LG05g11200	PLP2, phospholipase	biotic/abiotic stress response		
<i>Solanum lycopersicum</i> L.	Solyc08g006150	ChaC-like family protein	glutathione degradation	nCeO2 nLa2O3 nCuO	Pagano <i>et al.</i> , 2016
	Solyc03g081240	PRR5, pseudo-response regulator 5	biotic/abiotic stress response		
	Solyc10g005080	LHY1, Homeodomain-like superfamily protein	stress response		
<i>Glicine max</i> L.	Glyma19g45030	LHY1, Homeodomain-like superfamily protein	stress response	CdS QD	Majumdar <i>et al.</i> , 2019
	Glyma15g06800	PR1, pathogenesis-related gene 1	biotic/abiotic stress response		

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1134 Table 2. Principal evidence of the ENM biotransformation in plant observed by physiological,  
 1135 molecular and synchrotron-based analyses.

ENM	plant	physiological response	molecular response	techniques	biotransformation	reference
nCeO2	<i>Cucumis sativus L.</i>	nCeO2 exposure had no significant effects on the biomass production under both the +P and -P conditions. However, the uptake of Ce in the plants is different under the two conditions	-	TEM XANES NEXAFS	high stability	<i>Rui et al., 2015</i>
	<i>Lactuca sativa L.</i> <i>Cucurbita pepo L.</i> <i>Zea mays L.</i> <i>Glycine max L.</i>	biomass in the agricultural soil amended with biochar 600°C was largely unaffected	-	SEM μ-XRF μ-XANES	modified redox state, from Ce(IV) to Ce(III) low translocation from roots to shoots	<i>Servin et al., 2017</i>
nCeO2 nZnO	<i>Glycine max L.</i>	-	-	μ-XRF μ-XANES		<i>Hernandez-Viezcas et al., 2013</i>
nCeO2 nLa2O3	<i>Cucumis sativus L.</i>	nCeO2 had no phytotoxicity to cucumber at all tested concentrations, while nLa2O3 showed significant inhibition on root elongation, shoot elongation, root biomass, and shoot biomass, as well as induced more reactive oxygen species and cell death in roots	-	μ-XRF XAS	higher dissolution compared to nCeO2 moderate translocation from roots to shoots	<i>Ma et al., 2014</i>
nTiO2	<i>Cucumis sativus L.</i>	at all concentrations, nTiO2 significantly increased root length (average >300%)	-	μ-XRF μ-XANES	high stability	<i>Servin et al., 2012</i>
	<i>Cucumis sativus L.</i>	In nTiO2 treated plants, the chlorophyll content in leaves increased as the external concentration of NPs increased. nTiO2 treatments increased CAT activity in leaves.	-	μ-XRF μ-XANES FTIR	low translocation from roots to shoots	<i>Servin et al., 2013</i>
nAu	<i>Nicotiana tabacum L.</i>	leaf necrosis was observed after 14 days of exposure to 3.5 nm nAu	-	μ-XRF	high stability no changes in Au valence	<i>Sabo-Attwood et al., 2012</i>
nAg	<i>Lolium multiflorum L.</i>	nAg and ionic silver significantly reduced growth, resulting in shorter shoots and roots and lower biomass. The growth inhibition from nAg was stronger than that from AgNO3. Higher concentrations of AgNPs caused broken epidermis and rootcap. Cell structures were unaltered in AgNO3 treated roots.	-	μ-XRF XANES	low stability high translocation from roots to shoots	<i>Yin et al., 2011</i>
	<i>Lactuca sativa L.</i> <i>Cucurbita pepo L.</i> <i>Zea mays L.</i> <i>Glycine max L.</i>	fresh foliar biomass was unchanged. Chlorophyll a, chlorophyll b, carotenoid and pheophytin contents were not affect	-	SEM μ-XRF XANES		<i>Larue et al., 2013</i>
	<i>Medicago sativa L.</i>	-	-	TEM XRF		<i>Stegemeier et al., 2015</i>
nZVI	<i>Cucumis sativus L.</i>	nZVI treatments did not affected the biomass of plants in hydroponic or soil systems. Only nZVI treated plant shoots grown under hydroponic conditions exhibited increased biomass (15%). Chlorosis observed in the leaves of the control plants but not in the plants treated with nZVI	ATPase isoforms increased their expression in the roots of plant exposed to nZVI.	EXAFS	low stability limited translocation from roots to shoots modified particle structure	<i>Dwivedi et al., 2018</i>

1136 Table 2 continue in the next page ...

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ENM	plant	physiological response	molecular response	techniques	biotransformation	reference
nZnO	<i>Zea Mays L.</i>	-	-	$\mu$ -XRF XANES	low stability high translocation from roots to shoots	<i>Lv et al., 2015</i>
	<i>Zea Mays L.</i>	By the 7th day, the treatment of 9 nm nZnO and ZnSO <sub>4</sub> significantly reduced the dry weight of roots by 44% and 58% respectively, compared to the unexposed control plants. In general, ZnSO <sub>4</sub> treatment had the greatest effect on root biomass, followed by 9 nm nZnO and finally 40 nm nZnO	-	$\mu$ -XRF		<i>Lv et al., 2021</i>
nCuO	<i>Nicotiana tabacum L.</i>	When exposed to equivalent weight of Cu, nCu <sub>2</sub> O exhibited higher toxicity than nCuO, implying that the transformation may elevate the toxicity upon nCuO exposure	-	TEM XANES	low stability high translocation from roots to shoots consistent with an increased ion release modified redox state, from Cu(II) to Cu (I)	<i>Dai et al., 2019</i>
	<i>Lactuca sativa L.</i>	Cu exposure had limited impacts on lettuce biomass. For the unweathered exposures, only the root biomass of NP-exposed plants was less than in bulk treatment; no other tissue- specific differences were evident. In the W exposure, the total biomass ranged from 8.2 g (W NP) to 9.5 g (unexposed control); nCuO and ion-treated plant biomass was significantly less than the unexposed controls. With regard to individual tissues in the W exposure, there were no differences of significance in the root biomass.	The expression level of nine genes involved in Cu transport shows that the mechanisms of nCuO and bulk CuO response-accumulation are different from ionic Cu	$\mu$ -XRF XANES		<i>Servin et al., 2017</i>
	<i>Cucurbita pepo L.</i>	no impact on zucchini biomass, photosynthetic activity or cellular respiration.	RNA-seq analyses on vegetative and reproductive tissues highlighted common and nanoscale-specific components of the response. Mitochondrial and chloroplast functions were uniquely modulated in response to ENM exposure as compared with bulk and salt forms	$\mu$ -XRF XANES EXAFS		<i>Marmioli et al., 2021</i>
CdS QD	<i>Arabidopsis thaliana L.</i>	treatment with CdS QDs caused a slight stress that increased the biomass in the mutants, but not in the wt, while CdSO <sub>4</sub> caused modest phytotoxicity to both the wt and mutants	-	EXAFS	high stability limited ion release high translocation modification in bonds distance	<i>Marmioli et al., 2020</i>